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# THE DEMONSTRATION OF OESTRUS IN THE VITAMIN A-DEFICIENT RAT BY SUPRAVITAL STUDY OF THE VAGINAL SMEARS <sup>1</sup>

K. E. MASON AND E. T. ELLISON

*Department of Anatomy, Vanderbilt University School of Medicine, Nashville, Tennessee*

## TWO FIGURES

(Received for publication December 4, 1934)

In the preceding report (Mason and Ellison, '35), it was observed that regular oestrous cycles could be followed, by dry stained smears, in A-deficient rats until the variations in the daily smears became obliterated by excessive keratinization of the vaginal epithelium. That oestrous changes still occurred was indicated by the successful matings secured when normal males were placed with the deficient females, confirming similar observations made by Evans and Bishop ('22) and by Evans ('28).

Guttmacher ('26) observed that the cellular changes of oestrus could be readily followed in the normal rat by examination of the living cell types in lavages obtained by pipetting physiological saline, containing small amounts of neutral red, into the vagina. He observed that nucleated epithelial cells were sloughed into the vaginal lumen in prooestrus, cornified cells in oestrus, and active leucocytes were found in large numbers in metoestrus. As dioestrus progressed, the leucocytes became fewer in number and showed degenerative changes, indicating that the leucocytic invasion occurred chiefly at metoestrus. This same method has been successfully

<sup>1</sup> This investigation was aided by grants from the Commonwealth Fund and from the Division of Medical Sciences of the Rockefeller Foundation.



applied to the study of vaginal changes in the A-deficient rat by Aberle ('33).

Application of this technic, some 4 years ago, proved of great assistance in interpreting the cellular changes observed in the dry stained vaginal smears previously described (Mason and Ellison, '35). Its further application has made possible the demonstration of cyclic vaginal changes in the more advanced stages of A-deficiency, where the routine dry smear proved inadequate. The present report will deal with the results of the latter study, together with an analysis of the factors involved in the irregularity of the oestrous changes noted in rats suffering from severe depletion of vitamin A.

#### EXPERIMENTAL

The dietary and other general experimental procedures were essentially the same as those used in studies reported in the preceding report (Mason and Ellison, '35), and require no further elaboration at this time.

Supravital vaginal smears were made, following the technic of Guttmacher ('26), by pipetting into the vagina 1 cc. of a 1 to 10,000 solution of neutral red, made up in normal saline. The cellular suspension obtained was withdrawn by the pipette and placed on clean cover glasses. The latter were then inverted on specially prepared neutral red slides, as used in blood studies in this laboratory. The cover slips were rimmed with cedar oil and kept in a warm chamber at 37.5°C. while the counts were being made. Two hundred cells were counted and a general survey of the complete smear made in order to compensate for any clumping of cells.

##### *1. Changes in the supravital vaginal smears of A-deficient rats*

The cyclic changes in the vaginal smears of A-deficient rats up to the time at which cornification became complete and continuous have been fully described in a separate report (Mason and Ellison, '35). When this latter condition was reached, the supravital staining of the smear became particularly useful. The general appearance of these smears representative of severe A-depletion, will be briefly outlined.

In general, the daily sequence of events was represented by smears containing cornified cells only, for a period of 2 to 4 days, alternating with smears in which variable numbers of leucocytes and a few nucleated epithelial cells appeared intermingled with cornified cells, for a period of 2 to 4 days. The periods of prolonged and complete cornification resembled those observed in dry stained smears (fig. 14, Mason and Ellison, '35) the last day of which represented the period of true oestrus as demonstrated by the invariable acceptance of the male at this time. The periods of leucocytic infiltration were comparable to those observed in dry stained smears (figs. 12 and 13, Mason and Ellison, '35) before cornification became complete and continuous.

The latter type of smear showed certain variations during the interval between periods of complete cornification. During the early phases of this interval the leucocytes were particularly abundant and very active, as indicated by their capacity to take up neutral red. This we interpreted as representing the period of metoestrus. During the middle and latter part of this interval the leucocytes not only decreased in number, but showed much less evidence of activity and more of them appeared to be in a dying state. This stage was considered to represent the dioestrous interval, and was immediately followed by several days of complete cornification, during which no distinction could be made between the stages of prooestrus and of oestrus.

From what has been said above, it is apparent that the supravital method merely permitted more ready detection of the appearance of leucocytes which were intermingled with cornified cells<sup>2</sup> at periods when the process of cornification

\* The large cornified non-nucleated cells, observed in all stages of the cycle, contained within their cytoplasm large numbers of rod-like particles in active brownian movement. Application of Gram's stain to dried fixed smears of these cells has demonstrated that at least many of these particles were bacteria. Culture media inoculated with these cells have also demonstrated the presence of many organisms, largely of the colon bacillus type, either in the cells themselves or in the vaginal contents. Though such organisms are present in the vagina of normal rats, comparisons with similar cultures from the latter indicated that the bacterial flora was definitely increased in A-deficient rats.

had become so extreme that the leucocytes were not readily demonstrable in the dry stained smears.

## *2. Variability in length of the oestrous cycle*

Slight increases in the length of the oestrous cycle were sometimes observed in A-deficient rats in which increment in growth had ceased. During more advanced stages of A-deficiency, associated with marked xerophthalmia and loss of body weight, it was not unusual to observe cycles lasting from 8 to 20 days. Moreover, rats which failed to attain a weight of more than 100 to 110 gm. on the deficient diet usually exhibited a complete absence of oestrous activity, even though the deficiency symptoms were less severe than in other rats showing fair growth and only slightly irregular cycles. Frequently, this same phenomenon appeared during an unusually rapid growth response following administration of vitamin A to animals in a very depleted state.

Study of the vaginal smears demonstrated that the lengthening of the oestrous cycle was due, almost entirely, to prolongation of the dioestrous interval. The periods of complete cornification rarely exceeded 3 or 4 days in duration. In view of this apparent relationship between the lengthened oestrous cycles and associated growth disturbances of A-deficient rats, it seemed essential to analyze this problem more carefully by means of more carefully controlled conditions.

*a. Comparison of A-deficiency and inanition.* The oestrous cycles of A-deficient female rats were contrasted with those of littermate sisters fed the same diet made adequate by the addition of cod liver oil, but restricted in the amount given such that their growth rate was approximately the same as that of the former group (fig. 1). In order to maintain a moderately severe state of A-deficiency, without endangering the life of the animal, it was necessary to administer small doses of diluted cod liver oil, orally, at various times during the first 120 days of experiment (periods A and B). This amounted to a total of approximately 10 drops of cod liver oil for each of the three A-deficient rats. The latter exhibited

marked vaginal cornification and mild to moderately severe xerophthalmia from the 40th to the 120th day of experiment (period B), their oestrous cycles averaging 14.3 days in length, as compared with an average of 15.3 days for the 'restricted' controls during the same period.

The deficient rats were then given a single dose of three drops of cod liver oil on the 120th day; sufficient to produce a

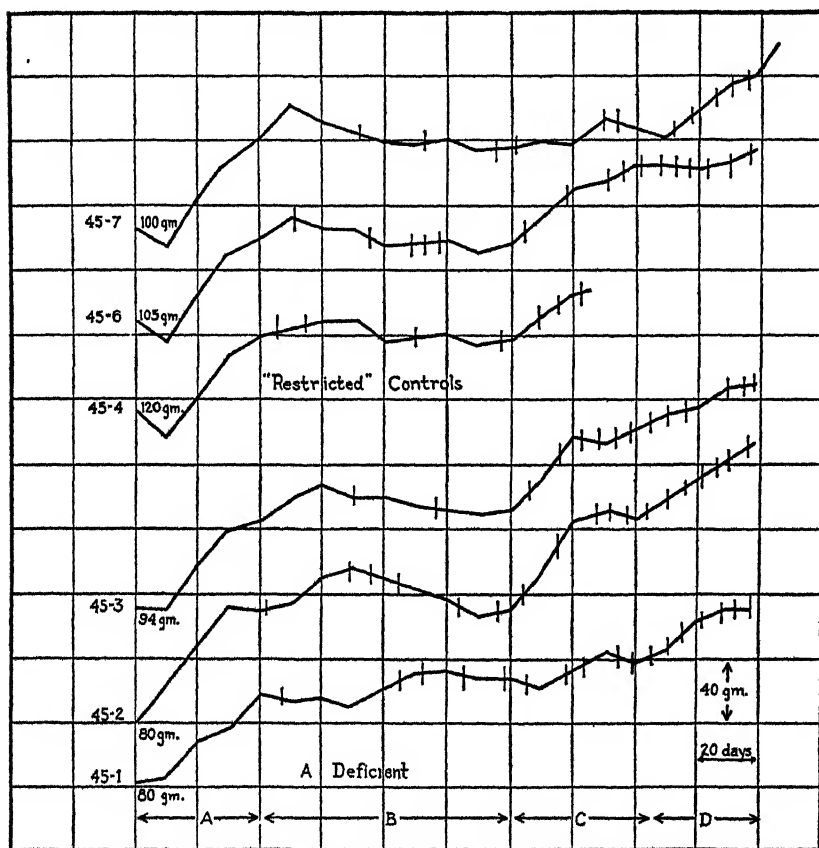


Fig.1 Showing the frequency of oestrous cycles in vitamin A-deficient rats and in littermate sisters fed an adequate diet restricted in the amounts given such that their body weights approximated that of the deficient animals. A, period prior to taking of vaginal smears. B, period of chronic A-deficiency in rats 45-1 to 45-3. C, period of mild A-therapy in deficient rats. Sufficient to stimulate growth, but scarcely adequate to repair eye changes. Vertical lines represent the time of appearance of the oestrous phase of the cycle.

moderate growth response, but inadequate to completely repair the severe xerophthalmia. During the 43 days following (period C), the cycles were somewhat shortened; more so than in the 'restricted' controls, apparently due to the fact that the additional food supplied the latter was not sufficient to produce the same growth response as that exhibited by the deficient rats.

During the remaining 35 days of the experiment (period D), the A-deficient rats were given 6 drops of cod liver oil, daily, and the controls maintained at approximately the same body weights as the former. The xerophthalmia disappeared in the deficient rats after from 12 to 18 days of therapy, and the growth response was marked. Toward the latter part of this period, oestrous cycles of approximately normal length became established. The average length of the oestrous cycles of the control rats during the periods B, C, and D (fig. 1) was 15.3, 12.2, and 7 days, respectively, as compared with 14.3, 7.4 and 6.5 days for the A-deficient rats during the corresponding periods. It is quite evident, from the foregoing, that the quality of body growth, rather than the lack of vitamin A per se, was responsible for the irregularity and lengthening of the oestrous cycle in the deficient rats.

*b. Combined A-deficiency and inanition.* Five pairs of rats from two litters were fed an A-deficient diet (diet 56) in carefully regulated amounts such that their body weights were maintained at levels of approximately 120, 150, 175, and 225 to 250 gm., respectively (fig. 2). As in the preceding series, daily weighing of the animals and of the food was necessary to maintain the rats at the specified levels of body weight, and small doses of cod liver oil were supplied occasionally to prevent the deficiency symptoms from becoming too severe.

Fig. 2 Showing A, the effect of different levels of growth restriction on the frequency of oestrus in A-deficient rats (first 147 days). The lengthening of the oestrous cycles is in direct proportion to the severity of the growth retardation and independent of the other manifestations of A-deficiency. B, the effect of A-therapy associated with 1) gradual growth retardation induced by restriction of daily food intake (rats 40-73, -74, -76); 2) maintenance at same level of body weight (rats 40-67, -68, -71); 3) rapid growth on unlimited food supply (rat 40-69). Last 50 days.

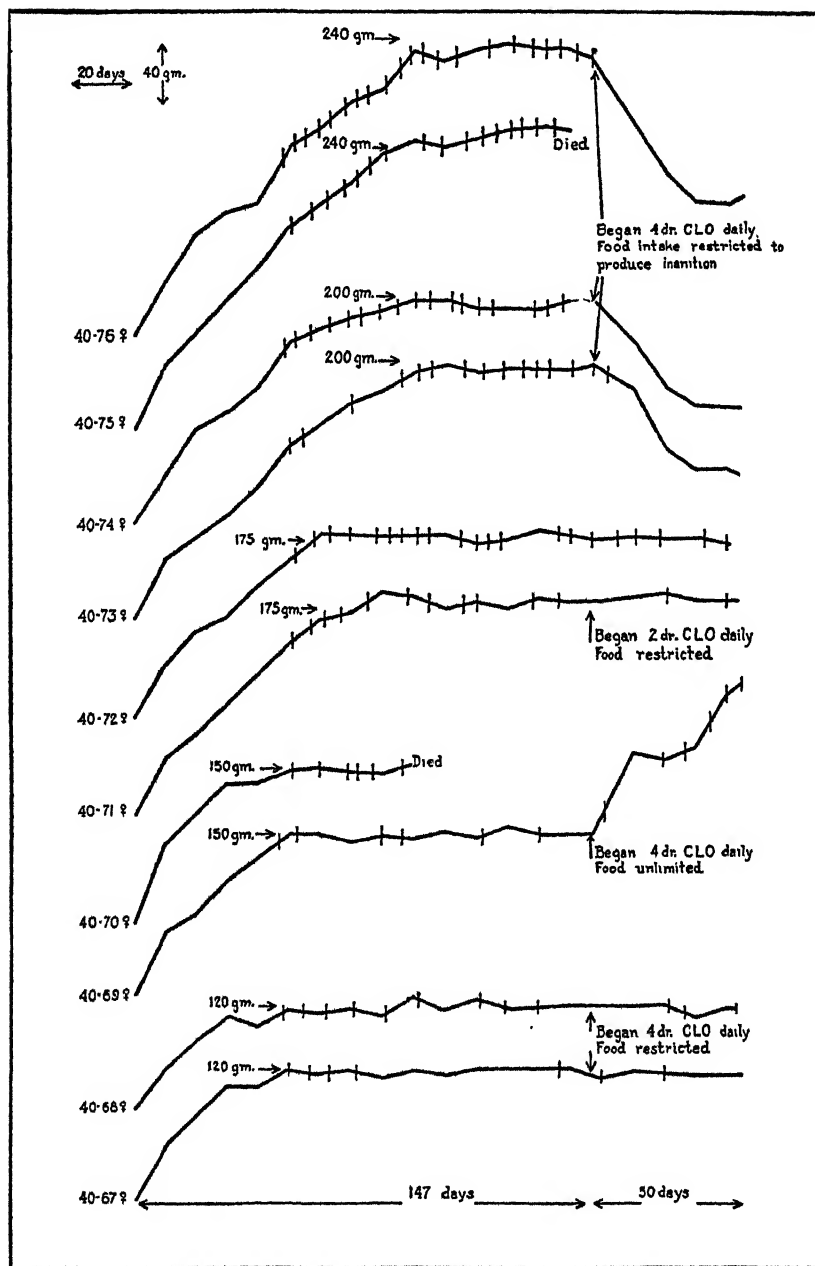


Figure 2

Vaginal smears were begun on the forty-seventh day of experiment and continued for 100 days before the experimental procedure was varied. During this period all the rats exhibited mild to moderately severe degrees of xerophthalmia and abnormal cornification of the vaginal epithelium. The eye conditions were somewhat more severe in the rats maintained at the 120 and 150 gm. levels, but, as was shown by subsequent treatment, this was not the factor responsible for the greater length of the cycles in these pairs. From the data shown in figure 2, it is quite evident that the frequency of the oestrous cycle was roughly proportional to the improvement in growth exhibited by the A-deficient rats. Moreover, if growth was sufficiently good, as in rats 40-73, 40-74, 40-75 and 40-76, the cycles were of normal length in spite of a rather marked and persistent cornification of the vaginal epithelium.

*c. Vitamin A-therapy in the presence of moderate and severe inanition.* Rats 40-67, 40-68 and 40-71 (fig. 2) were given daily doses of 4, 4 and 2 drops of cod liver oil, respectively, during the last 50 days of experiment. At the same time, their body weights were maintained at the same level as had existed before, by restriction of the food intake. Although xerophthalmia and abnormal vaginal cornification cleared up within 10 to 15 days, oestrous cycles were no more frequent than prior to the therapeutic treatment. In fact, one of these (rat 40-71) exhibited but two cycles, whereas its littermate sister (40-72), maintained at the same body weight and in a state of mild A-deficiency, had five cycles during this same period. It is apparent, from the above, that administration of vitamin A was incapable of correcting the prolonged oestrus in A-deficient rats if increased body growth was not permitted. On the other hand, the data on rat 40-69 adequately demonstrate the efficacy of vitamin A-therapy when supplemented with an unlimited supply of food.

The effect of more marked inanition during periods of vitamin A-therapy is shown by the data on rats 40-73, 40-74, and 40-76 (fig. 2). After showing cycles of approximately normal length for a period of 100 days, in spite of a mild

chronic xerophthalmia, these rats were given 4 drops of cod liver oil, daily, but their body weights were gradually diminished by a continuous reduction of the daily food intake. It will be seen that the degree of inanition was sufficient to completely suppress the oestrous cycles in these animals. After about 10 days, the vaginal smears of these rats consisted, for the most part, of small amounts of mucus and many leucocytes.

The data presented above demonstrate that the irregular and prolonged oestrous periods of A-deficient rats must be attributable to an indirect effect of the vitamin deficiency as reflected in the general nutritive state and quality of body growth of the experimental animals.

#### DISCUSSION

The studies presented in the present paper emphasize the importance of differentiating between the pathologic changes induced by lack of a particular vitamin per se and those due indirectly to other incidental conditions associated with the deficiency disease. In the alterations in the oestrous cycle of A-deficient rats, two separate factors have been clearly demonstrated. The specific effect due to lack of the vitamin factor is an excessive keratinization of the vaginal epithelium. The indirect effect, due to retarded body growth and generally lowered body state, is manifest in the irregularity and prolongation of the cyclic changes produced in the vaginal epithelium by hormonal influences.<sup>3</sup>

<sup>3</sup>A similar prolongation of dioestrus and increased lengthening of the entire cycle noted by Light ('27) in rats suffering from a deficiency of protein, of phosphorus and of vitamin A, seems best attributable to the general inanition of the experimental rats. Irregularity of oestrus has also been observed as a result of many other factors capable of inducing either hormonal imbalance or subnormal physical states of the experimental animal. Failure to recognize the effect of anorexia and physical debility upon the sensitive phenomenon of oestrus undoubtedly explains many of the contradictory statements in the literature regarding the effect of adrenalectomy, sympathectomy and other operative procedures upon the oestrous cycle.



Failure to recognize the importance of these two factors seems to best explain the failure of Parkes and Drummond ('26) to observe continuous cornification in the vaginal smears of their A-deficient rats, although they observed greatly prolonged cycles. Their animals usually weighed only about 40 gm. at 4 weeks of age, and rarely attained body weights of more than 100 gm. after 4 months of dietary treatment, and no mention was made of the existence of xerophthalmia. It would appear that their results were predominantly due to greatly retarded growth rather than to a direct effect of A-deficiency. It is apparently for the same reason that Coward ('29), and Coward, Morgan and Dyer ('30) were led to consider the vaginal cornification to be a rather unreliable criterion for diagnostic purposes.

We would like to stress the importance of using, for feeding experiments, animals which show unusually good growth and vigor during the weaning period. It has been our custom in most of our experimental work, to use only animals which have attained a body weight of 40 gm. or more at the twenty-first day of weaning. Utilization of inferior young has invariably led to atypical results, especially in studies concerned with the male or female reproductive system.

#### SUMMARY

1. The lavage method of obtaining smears of the cells of the rat's vagina, stained supravitaly with neutral red, makes possible the demonstration of oestrous cycles in vitamin A-deficient animals in which abnormal cornification has become too severe to permit their identification by dry stained smears.

2. The excessive keratinization, which more or less masks the underlying cyclic changes, is a primary response of the vaginal epithelium to lack of vitamin A.

3. The increased lengthening of the cyclic change is indirectly due to associated growth retardation and decline.

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# THE EFFECT OF RETARDED GROWTH UPON THE SEXUAL DEVELOPMENT OF RATS

S. A. ASDELL AND MARY F. CROWELL

*Animal Nutrition Laboratory, Cornell University, Ithaca, New York*

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The study of longevity on a retarded diet recorded by McCay ('35) has afforded means of obtaining data on the sexual development of rats. The rats used in the study were albinos derived from the Mendel colony. Although no outside blood has been brought into our colony for several years, no more effort has been made to render the animals homozygous than is usual in a colony used for nutrition work so that it is probable that considerable genetic variation may be expected in the stock. However, the fact that litter mates were distributed among the three groups set up should make the degree of variation in each group similar.

A brief recapitulation of the methods and objects of the experiment so far as they affect us may be made. Three groups of rats were set up; the first or control group were allowed to grow as fast as they could upon an adequate diet, both quantitatively and qualitatively. The second group were kept at a weight of 40 gm. and the third at a weight of 80 gm. until signs of failure occurred when both groups were allowed to rise 10 gm. in weight. This was continued until the rats were 766 days old when half the survivors were allowed an unrestricted diet, while at 911 days of age all the rats were unrestricted. The dietary deficiency may be regarded as solely one of calories. The animals were all kept isolated during the experiments; no matings were allowed so that the only observations which could be made were the time of opening the vaginal orifice and daily vaginal smears. After the

establishment of the vaginal canal, smears were taken daily until a rat showed six regular cornified smears at reasonably normal intervals, anything below 10 days being regarded as reasonable. Smears were then taken at 6-month intervals for a sufficient period, usually 6 weeks or more, to enable one to gauge the reproduction state of the animal at this time.

The circumstances under which the experiment was conducted allowed the following observations to be recorded.

1. Age and weight at the opening of the vaginal membrane.
2. Age and weight at first oestrus.
3. Regularity or otherwise of the oestrous cycles at intervals during life.

The results obtained under these headings are discussed separately for each group and compared.

#### *The control group*

These rats were allowed all they could eat of a qualitatively adequate diet. They may be assumed to have grown at an optimum rate and to have reached sexual maturity at the earliest age possible. We have no data on this point since it involves the ability to reproduce, which was not tested in this experiment.

Twenty-two rats were included in this group, but at the time the work was begun seven had open vaginas and their oestrous cycles were established. Also, one rat died immediately after opening, so her record was omitted from the record. The average age of opening for the remainder was  $53.5 \pm 0.19$  days, the spread being between 49 and 59 days. The average weight was  $136.7 \pm 2.1$  gm. with a variation from 120 to 165 gm. There is some evidence that the age of the rat is a more important determiner of the time of opening than the weight, but the number of cases discussed here is rather small. However, evidence pointing to the same result was obtained in the retarded groups so that this view is strengthened. If the rats in the normal group with ages of opening below the mean and those with ages above the mean are taken separately, the average weights of opening are 134.6 and 139.5 gm. respec-

tively, while for weights below and above the mean, the corresponding average ages were 53.6 and 53.4 days, a much closer agreement for the ages than for the weights.

We have been unable to find any records of the relation between age and weight at puberty for any species; all other records deal with age and the effect of various factors on the variability of the age. For rats, Evans and Bishop ('22 a) record that for 570 animals the average age at opening was 46.8 days, giving a considerably lower age for their strain, even allowing for the fact that some of our earlier opening rats were not recorded.

Our rats experienced their first oestrus, as judged by the vaginal smear, 1.8 days after opening, i.e., at 55.3 days. The delay in the Evans and Bishop rats was 0.5 day. One delay of 7 days greatly increased the average delay for our rats. On the whole, the behavior of the two strains is alike in this respect.

At the onset of their breeding powers all the control rats experienced regular cycles. In October 1931, when they were 11 months old, 21 rats of the group were alive; of these 13 were regular in their cycles, and 8 showed a continuously cornified smear. The average weight of the cornified rats was 275.9 gm. against 272.6 gm. for the normals.

In April 1932; age, 1 year 5 months, 19 survivors, 5 were regular, 10 cornified, 4 anoestrous. The normal rats weighed an average of 346.4 gm.; the cornified, 322.1 gm.; and the anoestrus, 375.8 gm.

In October 1932; age, 1 year 11 months, 12 survivors, 4 were regular, 1 cornified, 7 anoestrous. Average weights 297.3 gm., 320.0 gm., and 309.4 gm. respectively.

In April 1933; age, 2 years 5 months, 7 survivors, 2 were regular, 5 anoestrous. Average weights 263.0 gm. and 337.0 gm. respectively.

In October 1933; age, 2 years 11 months, there were 2 survivors, 1 being regular and the other anoestrous. Weights 280.0 gm. and 244.0 gm. respectively.

There is a marked tendency for the anoestrous rats to be heavier than those with regular cycles. The cornified rats are at first a little heavier than the regular ones, but, with increasing age, they lose this advantage. Probably the anoestrous rats experience the fattening which usually accompanies ovariectomy and are to be regarded as in a state of physiological ovariectomy. If the cornified rats are in a continuous state of oestrous activity, their relatively smaller weight is to be expected, but we have no data on the point, no activity studies having been made on our rats.

The frequency with which continued cornified smears occurred is surprising, 15 of 21 or 71.4 per cent showed a continued cornified smear at some time in their lives. This is probably due to the prevention of mating so that frequent cycles with ovulations occur. A thickening of the epithelium of the ovary and prevention of the rupture of follicles is a probable consequence. But we have no information on the number of rats which are allowed to reproduce which also show this continued cornification. This tendency is not inborn as a rule for all our rats had regular cycles at the beginning of their sexual life and some passed into this state later. Almost half the rats pass from the cornified state to an anoestrous condition while the other half tend to resume regular cycles. All patterns of behavior at the 6-month intervals were exhibited, i.e., cycles to cornified to anoestrus, cycles to cornified to cycles, and so on, except that when once a rat entered the anoestrous phase, she rarely showed further signs of sexual activity. So far as our experience shows, this condition is usually irreversible under our rather uniform conditions. Only one rat changed from cycles to anoestrus and then to cycles again. Her weight changes were 310 gm. to 460 gm. to 380 gm. which may be significant. While in the anoestrous state her weight was the greatest recorded during the experiment. Her return to the normal state was accompanied by a large loss in weight.

There is no evidence in our rats of a menopause at a given period of life such as is seen in man. The percentage of anoestrous rats at various ages was:

<i>Age</i>		<i>Number of rats alive</i>	<i>Per cent anoestrous</i>
	11 months	21	0
1 year	5 months	19	21.1
1 year	11 months	12	58.3
2 years	5 months	7	71.4
2 years	11 months	2	50.0

Cessation of breeding activity under our conditions is a gradual process.

### *The 40-gm. group*

The rats in the 40-gm. group were maintained at this weight until some of the retarded rats showed signs of failing or one died when the whole of the retarded groups were allowed to rise 10 gm. in weight. The diets used were adequate in protein, minerals and vitamins for growth at all ages but were deficient in calories. The method of raising the weight makes our data difficult to interpret since the slightest feeding of an extra amount of calories, or even a change in temperature leading to economy in utilization usually caused the animals to show a cornified or epithelial plus cornified smear (absence of leucocytes was used as the criterion of a heat period). Indeed this method was more accurate than the weighing of the animals as the histologist could often detect an oestrous smear before the balance showed a rise in weight. But, the rise having taken place, when the rats were maintained at a new weight level, cycles often ceased, so that often many weight increases took place, with a few cycles at each, before six regular cycles were experienced.

The weights at the time of the establishment of the vaginal orifice in these rats were much lower than in the rats of the other groups, but they were more uniform.

3 rats opened at 40 gm.

7 rats opened during the rise to 50 gm. or at 50 gm.

3 rats opened at 60 gm.

8 rats opened during rise to 70 gm. or at 70 gm.



On the other hand there was a much greater spread in the age of opening. The evidence shows that as the rats get older they open at a much lower weight than is normal. Age, therefore, is a more important consideration than weight in vaginal opening. The weight at opening in the three groups also declines progressively as the stunting is more severe, so that in the stunted groups, although the age at opening increases, opening does occur in spite of the lower weight.

The age at first oestrus averaged 357 days, and the weight 84.5 gm., a delay of 228.8 days and of 28.6 gm. over the controls. There is nothing in the evidence to indicate that capacity for experiencing oestrus is lost by the stunting. All the rats which died before their first oestrus were well below the maximum observed age at first oestrus, namely, 776 days.

The subsequent behavior of the rats is difficult to interpret. In all cases but one a considerable delay, usually from 200 to 300 days, was experienced before six regular cycles were recorded, and this usually happened when an extended rise in weight was occurring, so that there was time for the establishment of regular cycles.

At 1 year 11 months, 4 rats were regular the others decidedly irregular, not open, or not yet at the first oestrous period.

At 2 years 5 months, 8 were regular, 2 anoestrous after having been regular.

At 2 years 11 months, 4 were regular, 1 anoestrous, and 1 passing into anoestrus.

This is a better record than among the 80-gm. group, which, at this age, were all anoestrous or passing into this condition. None of this group showed a continued cornified smear at any time. This is in accordance with our experience in the 80-gm. group. We regard it as due to the smaller number of ovulations which had occurred in these retarded groups.

*The 80-gm. group*

The rats in the 80-gm. group were allowed to grow until they weighed 80 gm. after which they were kept at this weight until some failure occurred, when, as previously mentioned, all the retarded rats were raised 10 gm. in weight.

When observations were begun one rat of this group had an opened vagina. She was not considered in working out the averages for age at opening, which makes these averages slightly too high. This rat was 61 days old when she was observed. The average for the opening of her class (those opening at 80 gm.) was 63.4 days.

Of the twenty rats in the group: 10 opened at 80 gm., 6 opened during the rise to 90 gm., 4 opened at 100 gm.

The first heat did not usually follow the opening of the vagina. In fact in most cases a delay occurred; sometimes a very considerable delay and a rise in weight did not produce a cornified smear though it usually did. The average age at first heat was  $173.7 \pm 21.1$  days, and the average weight  $104.4 \pm 3.0$  gm. This represents an average delay of 93.8 days and of 17.4 gm. in weight between opening and first oestrus.

After the first cornified smear the usual behavior was to show two or more cornified smears and then to remain quiescent until another rise in weight was made to occur. When six regular cycles were completed the rats were regarded as being in a healthy reproductive state. This state was reached in a very variable time; the average was  $592.6 \pm 30.2$  days with weight  $158.7 \pm 4.39$  gm. Six rats died before this stage was reached. After six regular cycles had been noted, the rats usually continued to show regular behavior, but tended to become irregular as they grew older.

At 1 year 5 months, 2 rats were regular, both weighing 150 gm.

At 1 year 11 months, 3 were regular, 1 irregular, 2 anoestrous, 3 had not reached six cycles.

At 2 years 5 months, 4 were regular, 1 irregular, 2 anoestrous, 2 had not reached six cycles.

At 2 years 11 months, 3 were irregular, 1 becoming anoestrous, 2 were anoestrous.

No rats in this series became continuously cornified, a condition which was also noted in the 40-gm. group.

### *A small autopsy group*

In order to study the effects of this type of stunting on the reproductive tract, a group of six female rats was stunted by the methods used in the main experiment. These were killed at varying intervals.

A typical case is given here: Rat E63. Allowed to grow to 80 gm. and then allowed to rise gradually to 95 gm. Vagina opened at 78 days, weight 95.5 gm. steady. Killed at 106 days, no cornified smear. Reproductive tract infantile, uterine mucosa moderate in thickness, blood supply moderate and glands scanty. Ovaries, some follicular degeneration, moderate-sized follicles, no corpora lutea.

Three other rats with similar treatment produced a similar picture, but the degree of follicular degeneration was somewhat variable.

Rat E89. Killed 2 days after first cornified smear, 113 days after vaginal orifice established. Weight at killing 124.5 gm., rising. Uterus fairly well developed, glands moderate. Six recent corpora lutea were found in the two ovaries, but no ova were found in the tubes.

Rat E70. Killed 3 days after first cornified smear, 114 days after vaginal orifice established. Weight at killing 120 gm. on rise from 110 to 120 gm. Uterus fairly well developed, glands scanty. In the two ovaries were sixteen corpora lutea of at least two crops.

The results from these autopsied rats differ in no way from those which have been obtained by a number of other observers on the reproductive tract under the influence of inanition. The last case (E70) indicates that by our system of daily smearing occasional oestrous periods may be missed, though it is possible that with poor nutrition the full effects of ripening follicles on the vagina may not be exhibited.

*The males*

The conditions of the main experiment did not allow any experimental work to be done with the males. It was noted that all the males of the 40-gm. group developed a peculiar condition of the penis. This protruded from the sheath, and, instead of becoming pointed as in the normal adult, became bulbous with a flattened end. This prevented the return of the penis to the sheath and a condition resembling mild strangulation ensued, resulting in an aggravation of the condition. A similar condition occurred in a single male of the 80-gm. group and in none of the control group. The reason for the condition may be that male sex hormone was produced in these animals causing an hypertrophy of the penis at a time when the general body development was insufficient to accommodate it, thus resulting in a disharmony of the relative sizes of the parts (i.e., penis and sheath) and a distortion of the penis.

Two rats were grown under similar conditions to the experimental group and developed the condition of the penis described. They were killed when this occurred. Histological examination showed that the condition was due to oedema of the superficial layers of the corpus spongiosum which was well developed. The testes were almost normal, all layers of the seminiferous tubules were present and actual development of spermatozoa was occurring though not so frequently as in the controls. Only debris were found in the epididymes.

## DISCUSSION

The only similar experiments on the effect of partial inanition on the development of sexual function in rats seem to be those of Evans and Bishop ('22 b). These workers explored the results of several types of partial inanition, including one which was entirely an energy deficiency. The results showed the same delay in the opening of the vaginal opening and the same dissociation between the opening and the first oestrous period. Subsequent cycles were highly irregular, with long

intervals between them. In one group which was maintained at from 60 to 80 gm. for 375 days, the vagina opened on the average at the ninety-ninth day of life and no oestrous cycle was observed during the period of observation. Our rats, even of the 40-gm. group did not show this extreme result for three experienced their first oestrous periods at 42, 43 and 54 gm. respectively, their ages being 377, 101 and 106 days respectively. Possibly our method of increasing the diet so that 10 gm. were gained at one time is the reason for the difference, but on the other hand we found the occurrence of oestrus an extremely sensitive indicator of a gain in weight. We were able to deduce as a general rule, that, when the food was increased, even slightly there was no competition for the increased energy between the growth and the sexual functions. Each received its share of the nutrients until the growth had reached a stage at which all the energy was required for maintenance when there was none to spare either for growth or for sexual needs.

At the present time it is a logical conclusion from the evidence available to deduce that both growth and the sexual function are controlled by the anterior hypophysis. In the normal young animal the hypophysis releases growth substance only, and when the genetic growth stimulus slackens, then sexual stimulating substance is also released in gradually increasing quantities. This accounts for the onset of puberty and the subsequent development of sexual maturity, which, as Crew ('30) has shown, is a gradual process. Our work described in this paper has shown that, so far as the opening of the vaginal membrane and incidence of the first heat period are concerned, after a time, the age influence becomes so great that it overcomes the genetic growth impetus and causes the release, when made possible by more food, of the sexual stimulating substance at a lower weight than is normal. In other words, there is an aging of the animal which determines the mode of functioning of the hypophysis.

## SUMMARY

1. The oestrous behavior in the absence of mating was observed throughout life in a group of rats allowed to grow at an unrestricted rate, in a group retarded for a considerable period at 40 gm. and then allowed to grow slowly, and in a group similarly treated after an initial weight of 80 gm. The limiting factor was that of energy in the diet.

2. The average age at the opening of the vagina is recorded, also the weight at this time, for each group.

3. The age of opening of the vagina increases with the increased severity of treatment and the weight at opening decreases. Two lines of evidence indicate that age is a more important consideration than weight in determining the time at which vaginal opening occurs.

4. Increased severity of treatment increases the age and weight interval between the vaginal opening and the occurrence of first oestrus. These two events become entirely dissociated in the retarded rats.

5. In the control rats the cessation of sexual activity is a gradual process, not abrupt as in man. It is usually irreversible.

6. Anoestrous rats tended to be heavier than rats with regular cycles.

7. Seventy-one per cent of the normal rats presented continued cornified vaginal smears at some time of their lives, while this never occurred in the two retarded groups. This is probably associated with the frequency of ovulation. Cornified rats tended to weigh less than those with regular cycles.

8. The occurrence of an oestrous smear is a very delicate indication of an increased supply of energy.

9. When more energy is supplied it is used for both growth and sexual needs; no competition is shown between these demands. But when growth has proceeded so far that all the energy available is needed for maintenance both growth and sexual activity cease.

10. The males of the 40-gm. group show a deformity of the penis, the etiology of which is discussed.

11. The bearing of 3 and 9 on the mode of action of the anterior hypophysis is discussed.

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# STUDIES OF CRYSTALLINE VITAMIN B

## IV. INJECTION METHOD OF ASSAY <sup>1</sup>

MARION AMMERMAN AND ROBERT E. WATERMAN

*Department of Physiological Chemistry, Teachers College, Columbia University*

### ONE FIGURE

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It has been found advantageous in testing the potency of vitamin B(B<sub>1</sub>) materials in this laboratory to use a variety of methods. In the earlier stages of work, when the emphasis was upon the resolution of vitamin B complex into its several factors, the activity of the fractions was determined by feeding experiments on rats and pigeons and later by feeding tests on rats only. However, during the past 2 years the principal objective has been the isolation of vitamin B(B<sub>1</sub>) and for this purpose it was found advisable to adopt a quicker method of test as a guide for chemical procedures and one which is as specific as possible for the antineuritic factor alone. Smith's ('30) curative injection method presents an effective means of rapid assay. With slight modification it can be used on crude fractions. Over 2000 rats have been used in the testing of 500 preparations and the method has always proved reliable in estimating relative potencies.

The availability of crystalline vitamin B (Williams, Waterman and Keresztesy, '34) now affords an opportunity for noting the effect of specific doses of a single chemical entity and so calibrating this testing method.

<sup>1</sup> The first three papers in this series appear in J. Amer. Chem. Soc., 1934, vol. 56, p. 1187, 1935, vol. 57, pp. 517 and 536. In this series the designation B<sub>1</sub>, preferred by the authors, has been used when consistent with the editorial policy of the journal involved.



*Breeding and care of experimental animals*

The rats used in this work were from a colony of albinos maintained on a stock diet consisting of one-third whole milk powder and two-thirds ground whole wheat with the addition of 1 per cent  $\text{CaCO}_3$  and 1 per cent  $\text{NaCl}$ . About 1 gm. of raw beef was given to each rat three times a week. The young rats were kept on this diet until they weighed 100 to 125 gm. which occurred at 40 to 55 days of age. They were then placed in separate cages and given the following depleting diet:

	<i>Per cent</i>
Casein (technical)	16
Osborne and Mendel salt mixture	4
Cod liver oil	2
Unfiltered butter	9
Cornstarch	60
Autoclaved dried bakers' yeast	9

The dried bakers' yeast was autoclaved for 5 hours at 15 pounds' pressure at its natural pH, and then finely ground. This diet, which is a modification of the Sherman-Spohn diet, contains a small amount of the antineuritic vitamin. This has been found to be necessary to avoid inanition and to prolong the life of the rat sufficiently to permit the development of distinctive paralysis. Heyroth ('32) and Birch and Harris ('34) have also purposely used a depletion diet containing small amounts of vitamin B in their injection assays. This is contrary to Smith's view ('33), but he ignores the presence of small amounts of antineuritic vitamin which we (Williams, Waterman and Gurin, '32) have found to be present in autoclaved brewers' yeast such as he uses as part of his diet. The best polyneuritis-producing diet would be one supplying a precisely controlled small amount of B, from our experience an amount equivalent to about 0.5  $\gamma$  per day of the crystalline vitamin. The rats gain in weight for about 2 weeks on the depletion diet, after which there is a gradual decline. In about 5 to 8 weeks, when the animals weigh 70 to 90 gm., paralysis occurs. This condition is characterized by incoordination, spasticity, and rolling movements of the head and

body. Of a group of 375 rats given this depletion diet, 70 per cent developed distinctive cases of paralysis. The remaining 30 per cent died without our observing paralytic symptoms and death was ascribed to inanition, urinary hemorrhage<sup>2</sup> or to paralysis so severe as to cause death before the animal could be used for test.

### *Technic*

The material to be tested is injected from a tuberculin syringe through a 26 gauge needle into the fleshy part of the rat's hind leg. This procedure is more expeditious, requires less skillful manipulation than intravenous injection and only one operator is necessary. The solution is roughly adjusted to pH 4.0 to 6.0, but no especial care is taken in regard to salt concentration. As much as 0.75 cc. of liquid containing as high as 10 per cent solids can be introduced into each leg subcutaneously with satisfactory results. With high concentrations temporary soreness is noted at the point of injection. Lameness of the injected leg occasionally appears.<sup>3</sup> The effect of the injected material can be noted within 12 to 48 hours. When an adequate dose is administered, the symptoms of paralysis disappear entirely with an accompanying gain in weight. If the dose injected is grossly inadequate, the symptoms are not alleviated and may become definitely worse and there is generally a loss in weight. If the injected dose is barely adequate, a partial cure may result. In such border-line cases, the animals must often be re-examined and weighed on the third day in order to observe

<sup>2</sup> Occasionally, toward the end of the depletion period, some of the rats show severe hemorrhage in the urinary tract. Such a condition occurs in both males and females and is evidently not of genital origin. This hemorrhage, except in one case, has invariably proved fatal in a very few hours. Despite the fact that several animals may develop this condition at the same time followed by relatively long periods during which no cases are noted, we have no other reason to suspect that the disorder is infectious. It is not known whether this is a specific disease or one of the manifestations of extreme depletion.

<sup>3</sup> On the suggestion of Dr. Hans Molitor, of Merek & Co., subcutaneous injection in the back of the animal has been tried with marked success. The difficulty of distinguishing between leg soreness and paralysis is avoided and larger volumes can be injected.

retarded responses and so secure an indication of the degree of potency of the dose. With minimal curative doses paralysis recurs in 5 to 10 days after successful treatment. As a result of an extremely large dose, animals have remained cured for as long as 32 days. Surviving animals that are not cured by the first injection are used again after the lapse of 1 day. Those that are cured or definitely improved are kept on the depletion diet until paralysis recurs and are then reinjected. In this way the same rat can be used several times. Rats have been used for as many as ten successive tests with practically no change in response. A survey of injections during a given period of use of the method showed that 614 tests were made on 257 rats—an average of 2.4 tests per rat. Twenty-five per cent of the deaths followed trial doses which subsequent tests proved inadequate.

#### *Individual response*

A curative dose has arbitrarily been called the minimum amount capable of effecting cures of which at least two out of three shall be complete. A standard might with equal validity be set at which a dose would be the minimum amount completely curing every surviving animal injected. This would require more animals. According to our procedure, partial cures are helpful in obtaining reasonably precise estimations with a minimum number of injections.

A small percentage of the animals injected with doses, proved to be curative for other animals, dies within the first 24 hours. Such results are discarded as death is considered to be due not to the inadequacy of the dose but to a paralytic condition too advanced to be alleviated. It has been found that deaths of this sort can be eliminated to a great degree by the early detection and treatment of the paralytic symptoms. Sometimes a dose found curative for several animals produces mere improvement rather than cure in an individual rat; with such a dose completely negative results are never encountered in surviving rats. On the other hand, in no case has a rat recovered after receiving a preparation later found to contain little or no antineuritic vitamin.

The individual responses obtained from 181 injections of varying amounts of crystalline vitamin B hydrochloride are given in table 1. From these data it has been concluded that the curative dose according to our standards is 5  $\gamma$ . Seven and one-half  $\gamma$  is the minimum dose which completely cures practically every animal.

### *Consistency of results*

As in all forms of animal tests the precision of the method leaves something to be desired. The degree of consistency obtainable may be judged from the results on seven lots of pre-

TABLE 1

*Individual responses on various dosages of crystalline vitamin B hydrochloride*

DOSE ( $\gamma$ )	NUMBER OF ANIMALS			
	Cured	Partially cured	Not cured	Died <sup>1</sup>
2	0	2	2	3
3	2	4	0	2
4	8	10	3	12
5	33	17	2	20
6	10	8	0	2
7.5	17	2	0	6
10	9	0	0	7

<sup>1</sup> Discarded due to death in less than 24 hours.

sumably pure vitamin B (table 2). While relatively few tests have been made on some of the lots, 5  $\gamma$  is considered the approximate dose in each case.

### *Duration of cure*

Kinnersley, Peters and Reader ('28) have extensively used duration of cure as a means of evaluating the potency of antineuritic products. Their test animal is the pigeon and their 'day dose' is obtained by dividing the dose given by the number of days the bird remains free from polyneuritis. To determine whether such a method could be used in connection with the injection technic on rats, varying doses of a

single lot of crystalline B were administered to polyneuritic rats and the length of time before the recurrence of paralysis was noted. The results as given in table 3 show that the

TABLE 2

*Consistency of results on various lots of crystalline vitamin B hydrochloride*

LOT	DOSE ( $\gamma$ )	NUMBER OF ANIMALS			
		Cured	Partially cured	Not cured	Died <sup>1</sup>
A	4	1	0	2	0
	5	3	0	0	0
	7.5	1	0	0	0
	10	1	0	0	0
B	4	1	0	1	0
	5	2	1	0	1
	7.5	1	0	0	0
C	4	0	3	0	3
	5	5	2	1	5
	7.5	4	0	0	1
	10	8	0	0	7
D	2	0	2	2	3
	3	1	3	0	2
	4	4	1	0	0
	5	6	6	0	3
	6	6	2	0	1
	7.5	11	2	0	5
E	3	1	1	0	0
	4	0	2	0	2
	5	7	3	1	8
	6	1	0	0	1
F	4	2	4	0	7
	5	6	4	0	2
	6	3	2	0	0
G	5	4	1	0	1

<sup>1</sup> Discarded due to death in less than 24 hours.

duration of cure is a function of the dose but that the proportionality is far from exact. Our results are plotted in figure 1 and show a remarkable parallelism with the results

of Birch and Harris ('34) obtained from feeding various vitamin B preparations by mouth to depleted rats.

*Usefulness of method.* In the present state of uncertainty regarding the various factors in the B complex, feeding tests

TABLE 3  
*Effect of size of dosage of crystalline vitamin B on duration of cure*

DOSE ( $\gamma$ )	DURATION OF CURE IN DAYS	
	Individuals	Average
5	5, 7, 6, 5, 8	6.2
7.5	5, 8, 10, 6	7.3
10	8, 9, 7, 13, 9, 8, 6, 12	9.0
15	9, 8, 9, 14, 11, 6, 13, 10, 14	10.4
25	13, 11, 15, 17, 14, 18, 14	15.3
35	19, 16, 17, 25, 18, 22, 27	20.6
50	23, 23, 26, 21, 30, 28, 32	26.3

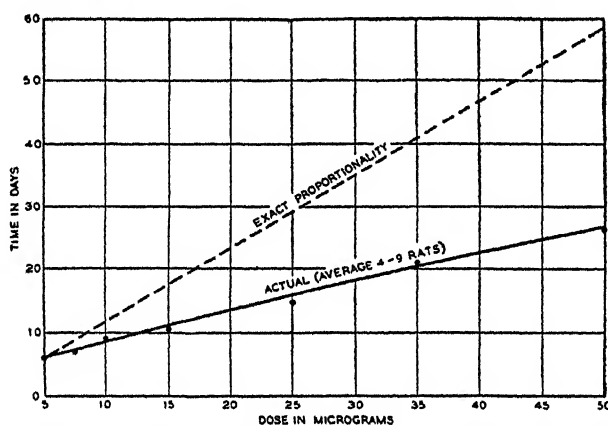


Fig. 1 Duration of cure of polyneuritic rats as a function of size of dose of crystalline vitamin B.

are necessarily open to question because the effect of over- or under-doses of any one of the other B factors may well have a profound effect on the rate of growth which is always used as a part of the critical data in such B assays. The injection technic on polyneuritic animals seemingly measures but one thing—the B content, and when the injections are

made subcutaneously relatively crude preparations can be used. This method is much to be preferred for the assay of therapeutic preparations. Liquid foods also are easily and accurately assayed in this manner. By developing a standardized extraction technic, it seems probable that practically all of the B in a solid foodstuff could be put into a solution suitable for injection. In this way a modification of the Smith injection method could be of practical use in the assay of foods, concerning the B content of which the present literature is grossly misleading on account of earlier failure to distinguish B from other growth promoting elements of the B complex.

The authors gratefully acknowledge generous financial assistance from the Carnegie Corporation of New York through the Carnegie Institution of Washington. This and the two succeeding papers are a part of the general study of vitamin B being carried out under the direction of Mr. R. R. Williams, to whom we wish to express our thanks for helpful criticism and advice.

#### SUMMARY

1. A modification of the Smith injection technic for vitamin B assay is described.
2. The method is quick and convenient and, for a biological assay, reasonably accurate.
3. Under the response standard established, the dose of crystalline vitamin B hydrochloride is 5  $\gamma$ .

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# STUDIES OF CRYSTALLINE VITAMIN B

## V. THE EFFECT OF GRADUATED DOSES ON GROWING RATS

ROBERT E. WATERMAN AND MARION AMMERMAN

*Department of Physiological Chemistry, Teachers College, Columbia University*

### SEVEN FIGURES

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Nutritional literature is replete with studies on the growth of young rats as effected by addenda of varying amounts of specific foodstuffs. There are few data on the growth attained when the addendum to a B-deficient diet is a vitamin in its pure form. The method of isolation of the antineuritic vitamin recently developed by Williams, Waterman and Keresztesy ('34) has made ample supplies of crystalline vitamin B hydrochloride available for study of the quantitative aspect of the effect of this vitamin in the diet.

### EXPERIMENTAL METHOD

The animals used were from a stock colony of albino rats maintained on a diet consisting of two-thirds ground whole wheat and one-third whole milk powder plus 1 per cent  $\text{CaCO}_3$  and 1 per cent  $\text{NaCl}$  with the addition of 1 gm. of raw beef per rat three times a week. At the age of 28 days, those rats which weighed 40 to 60 gm. were put in individual cages and given ad lib. the Chase modification of the Sherman-Spohn diet (Chase and Sherman, '31) consisting of 18 per cent B-free casein, 4 per cent Osborne and Mendel salt mixture, 2 per cent cod liver oil, 8 per cent butter fat, 53 per cent corn-starch, and 15 per cent dried autoclaved bakers' yeast. The bakers' yeast was neutralized with 0.1 N  $\text{NaOH}$  to pH 6.7 to

7.0, autoclaved at 15 pounds' pressure for 6 hours, dried at room temperature and finely ground. When possible, litter mates were put into test groups of two males and two females. After 15 to 17 days, the weights of the animals declined and addenda of crystalline B were started. The vitamin was fed every 5 days by mouth in amounts equivalent to daily doses ranging from 0.5  $\gamma$  to 160  $\gamma$ . (Studies are now in progress to determine the difference in growth attained by feeding addenda daily rather than in 5-day portions.) The crystals were dissolved in dilute alcohol and the solution pipetted into shallow dishes. The rats usually drink such a solution with avidity. Sometimes it was necessary to deprive the animals of water or to add a pinch of sugar to the solution in order to insure complete consumption of the supplement. The rats were weighed at 5-day intervals over a period of 45 days on the addenda. A control group of four rats was run on this diet whenever new lots of raw materials were used. Controls die in 28 to 40 days and rarely show typical paralysis, death being attributed to general inanition.

#### *Growth on assay doses*

Various investigators have proposed methods based on the rate of growth for assaying the B content of foods. Two standards now in use by which vitamin content is judged by growth rate are the Chase and Sherman ('31) and the Chick and Roscoe ('29). The former unit is that amount of vitamin B necessary to induce a gain of 3 gm. per week during an experimental period of from 4 to 8 weeks and the latter unit is that amount of vitamin B which results in a weekly rate of gain of 10 to 14 gm. The International unit is that amount of vitamin necessary to produce a rate of growth equal to that obtained by the daily administration of 10 mg. of 'International activated clay' to growing rats. Insufficient work has heretofore been done to indicate clearly the quantitative relationship of these different units of growth. It was therefore thought desirable to determine the relationship between the rate of growth and the size of dose under circumstances

where the basal diet is constant and the addendum consists of pure vitamin B without the complication of other factors. The results obtained in the dosage range of the usual B test will be discussed first.

All the animals in two groups of four fed at the level of 0.5  $\gamma$  of crystalline B hydrochloride died in less than 45 days with true paralysis. The behavior of these animals as contrasted to that of the control rats on the B free diet which died without paralysis is regarded as evidence for the necessity of including small amounts of B in diets designed for the production of typical polyneuritis (Ammerman and Waterman, '35). One  $\gamma$  was the lowest dosage on which all of a

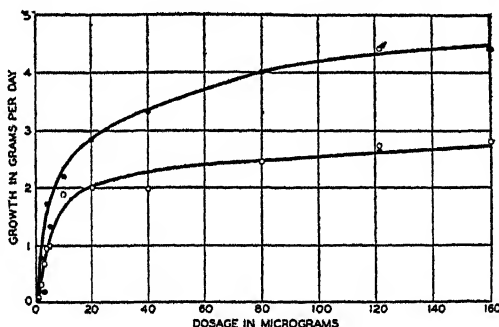


Fig. 1 Growth rate of young rats as a function of the size of dose. The period covered is 45 days following a 15- to 17-day depletion of 28-day-old rats. The dosage is the daily amount of crystalline vitamin B hydrochloride each rat received in 5-day portions during the 45-day test period.

single group of four rats survived the test period. Sherman-Chase unit growth occurred between 2  $\gamma$  and 3  $\gamma$  (fig. 1). Definite cases of paralysis have been encountered in animals receiving 1  $\gamma$  and even 2  $\gamma$  daily and one death, possibly due to B deficiency, occurred on the 3  $\gamma$  dosage. The 4  $\gamma$  level is the lowest which showed no suggestion of deficiency disease, the growth being about 1 gm. per day. Chick and Roscoe unit growth is not attained until a level somewhere between 5 and 10  $\gamma$  is reached. On rather incomplete evidence, we have provisionally set the B content of the Chick and Roscoe unit as 7.5  $\gamma$ . In comparison, 10 mg. of International standard

clay daily gave, with our diet, an average growth of 0.8 gm. per day for two males and two females, corresponding to the 5  $\gamma$  level of crystalline vitamin. In the preceding paper of this series, the curative dose by the Smith injection technic was set at 5  $\gamma$ . Each of the better known units may now be stated in terms of one another and also in terms of pure

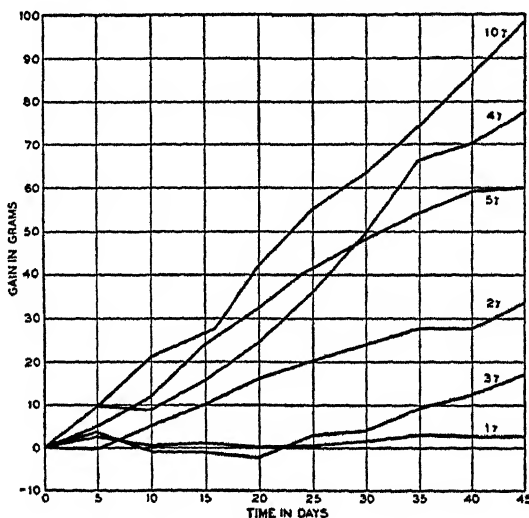


Fig. 2 Average growth curves of male rats receiving moderate amounts of crystalline vitamin B. The addenda were given every 5 days. The chart shows the intakes of vitamin calculated on a daily basis. The addenda were started after 28-day-old rats had been depleted 15 to 17 days on a B free diet.

vitamin B hydrochloride. This tentative equivalence is as follows:

- 1 Chick and Roseco unit = 7.5  $\gamma$  = 3 Sherman-Chase units
- 1 Smith unit<sup>1</sup> = 5.0  $\gamma$  = 2 Sherman-Chase units
- 1 International unit = 5.0  $\gamma$  = 2 Sherman-Chase units
- 1 Sherman-Chase unit = 2.5  $\gamma$

<sup>1</sup> Under an interpretation of the Smith method which sets the curative dose as that amount of vitamin which effects a cure in every successful injection, the unit would be approximately 7.5  $\gamma$  or three Sherman-Chase units.

However, attention is called to a certain lack of consistency in the growth response of different groups of our animals in spite of the fact that our stock colony has been inbred and maintained on the same diet for many years. This incon-

sistency is more conspicuous over the lower range of dosages (figs. 2 and 3) and reflects itself in variation from animal to animal at any given level, as indicated in table 1. This inconsistency of growth response is a weakness in any method of assay which depends on mere measurement of growth and is partly circumvented by the use of the International method providing the growth on an unknown is compared at the same

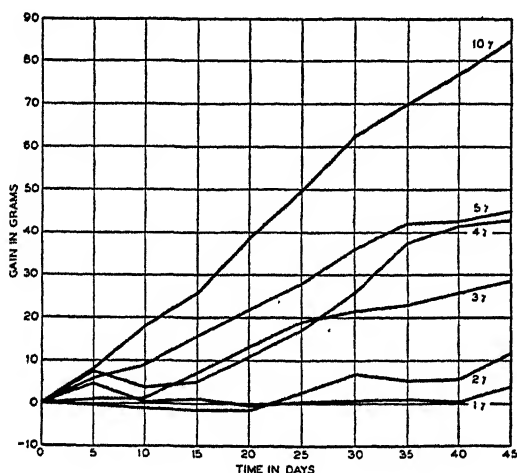


Fig. 3 Average growth curves of female rats receiving moderate amounts of crystalline vitamin B. The addenda were given every 5 days. The chart shows the intakes of vitamin calculated on a daily basis. The addenda were started after 28-day-old rats had been depleted 15 to 17 days on a B free diet.

time with growth on the standard with matched litters under identical conditions. This presupposes, of course, that the standard is constant. It will be more satisfactory to set crystalline B as the standard when enough of this material becomes available for universal calibration.

#### *Duration of cure*

At the end of the test period the surviving animals were given the polyneuritis-producing diet used in our injection assays (Ammerman and Waterman, '35) and the length of time before the onset of paralysis or death was noted. These data appear in table 1 and the average duration of time before

TABLE 1  
*Actual weights of individual animals*

DOSE (γ)	MALES					FEMALES				
	No. of rat	W <sub>1</sub>	W <sub>2</sub>	W <sub>3</sub>	E	No. of rat	W <sub>1</sub>	W <sub>2</sub>	W <sub>3</sub>	E
0.5	23431	40	62	D	D	23282	45	61	D	D
	23280	44	69	D	D	23279	48	76	D	D
	23281	44	76	D	D	23417	48	67	D	D
	23399	42	68	D	D					
	23397	45	62	D	D					
1.0	22771	45	53	45	3	22774	42	54	47	4
	22772	44	57	56	6	23807	40	63	69	11
	22773	44	55	48	4	23808	46	63	76	21
	23805	45	60	78	8					
2.0	21620	55	71	79	6	21621	47	40	45	4
	22094	46	74	95	9	21622	54	50	D	D
	22095	43	68	128	9	21623	56	64	57	2
	22096	44	73	118	13	22085	48	56	70	12
	22610	50	59	93	11	22602	52	65	76	13
						22607	59	81	121	15
3.0	22070	43	51	76	7	21298	48	49	76	21
	22071	45	45	65	3	21319	45	49	101	N
	21747	54	69	77	8	21320	46	51	96	23
	21748	56	70	D	D	22084	48	54	72	11
	21749	51	62	72	5	22303	44	46	46	8
						21750	52	68	66	10
4.0	22498	54	87	136	20	22504	51	68	144	24
	22499	46	68	180	22	20319	46	45	74	10
	22501	58	75	161	24	20320	57	58	83	10
	20317	53	51	123	19					
	20318	52	54	123	17					
5.0	20862	46	64	105	34	20864	43	61	94	38
	20863	42	61	128	49	21567	49	66	122	34
	20865	46	63	117	39	21568	51	72	119	17
	21565	54	81	140	18					
	21566	55	86	163	N					
10.0	21313	55	61	154	26	21314	49	56	116	24
	22604	59	79	170	26	21315	54	51	138	30
	22603	59	72	172	30	21316	49	55	117	28
	23125	44	65	137	24	22605	49	62	147	42
	23184	53	82	198	24	22608	62	84	182	32
						23126	42	64	147	36
						23186	43	51	169	47
20.0	21318	48	63	207	31	21336	49	53	131	32
	21333	53	54	177	27	21335	48	55	140	24
	23232	51	76	216	44	23234	49	83	164	44
	23233	47	69	180	28	23235	47	74	186	57
40.0	21728	47	63	222	38	21726	48	52	140	38
	22080	49	60	193	30	21727	48	50	140	30
	22081	55	68	219	40	21729	49	47	132	29
	22082	55	65	219	40	22083	48	56	139	49
80.0	22321	40	66	253	58	22324	41	66	176	48
	22322	40	62	249	43					
	22323	40	57	218	40					
160.0	22500	52	71	252	C	22503	52	69	190	C
	22502	55	77	292	C	22505	50	56	185	C

D = Animal died before completion of test. C = Animal being continued beyond normal test period. N = Data not available.

Column W<sub>1</sub> = Weight at weaning (28 days old). Column W<sub>2</sub> = Weight at start of test (28 to 33 days old). Column W<sub>3</sub> = Weight at end of test (73 to 78 days old). Column E = Time in days between end of test and onset of

## ERRATA

Volume 10, page 40. The second paragraph of the notes explanatory to table 1 should read as follows:

Column  $W_1$  = Weight at weaning (28 days old). Column  $W_2$  = Weight at start of test (43 to 48 days old). Column  $W_3$  = Weight at end of test (88 to 93 days old). Column E = Time in days between end of test and onset of paralysis or death.





death or paralysis of the several animals at each dosage level is given graphically in figure 4. In this curve the duration of time is the number of days between the last dose of vitamin and the onset of polyneuritis or death and the dosage given is the actual amount of vitamin administered at 5-day intervals. The results indicate that the duration of time bears a straight line relationship to the size of dose up to about 25  $\gamma$ . Beyond that level, the curve bends indicating a poor conservation of surplus vitamin in the animal organism.

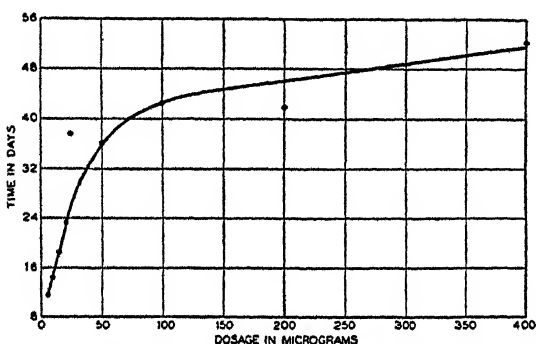


Fig. 4 Duration of effect in relation to size of vitamin B dosage. The time is the period from the last administration of the vitamin to the onset of paralysis or death. The dose is the actual amount of crystalline vitamin received.

#### *Growth on relatively high doses*

The literature contains many indications that a superior performance ensues from the administration of amounts of vitamin B larger than those requisite for the prevention of polyneuritis. There are also numerous suggestions, especially in the exploitation of commercial vitamin-containing products, that still larger amounts of vitamin contribute to general well-being. However, concrete evidence has heretofore been lacking that the effect of multiplication of the dosage extends far beyond the customary range of intakes. The continued upward sweep of the growth curves in figures 5 and 6 as the dosage levels range through 10, 20, 40, 80 and 160  $\gamma$  constitutes evidence of a remarkable stimulation of metabolic processes as reflected in growth. The rats receiving 160  $\gamma$  per day were continued on addendum beyond the

regular 45-day experimental period. Figure 7 gives the growth results on these animals up to an age of 233 days.

In these experiments there is little possibility that enhanced performance can be due to other vitamin factors in the ad-

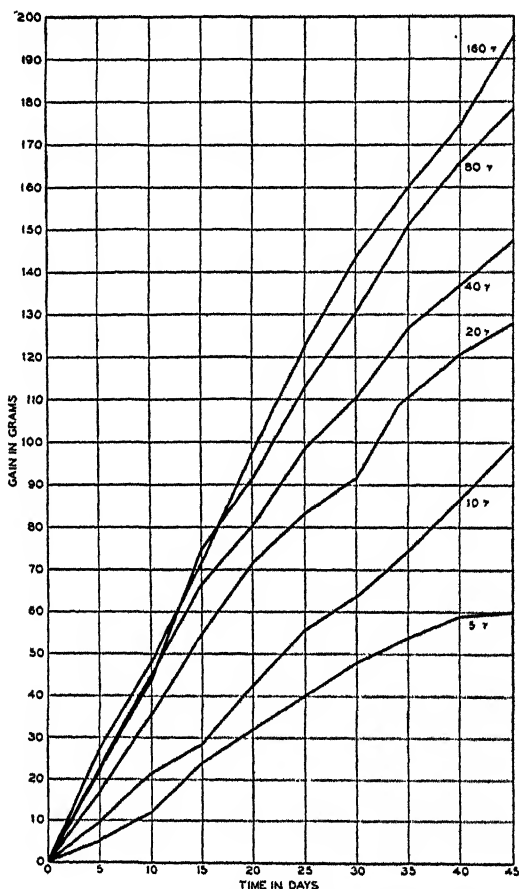


Fig. 5 Average growth curves of male rats receiving large amounts of crystalline vitamin B. The addenda were given every 5 days. The chart shows the intakes of vitamin calculated on a daily basis. The addenda were started after 28-day-old rats had been depleted 15 to 17 days on a B free diet.

dendum as is the case when the addendum consists of a crude product. Vitamins A, D, G, etc., which quite possibly are present in sub-optimal amounts remain constant increasing only with the food intake yet the growth at the higher

levels of B dosage approaches the best obtainable with rich mixed diets (Osborne and Mendel, '33). A possible explanation of this remarkable growth stimulation is that the crystals contain  $B_4$  as an impurity. This is not believed to be the

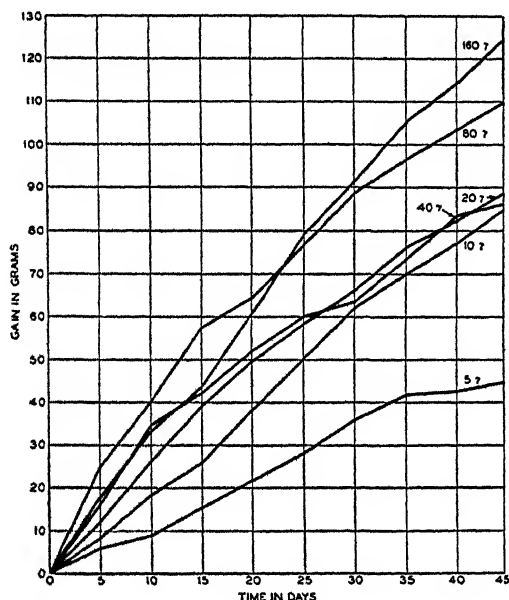


Fig. 6 Average growth curves of female rats receiving large amounts of crystalline vitamin B. The addenda were given every 5 days. The chart shows the intakes of vitamin calculated on a daily basis. The addenda were started after 28-day-old rats had been depleted 15 to 17 days on a B free diet.

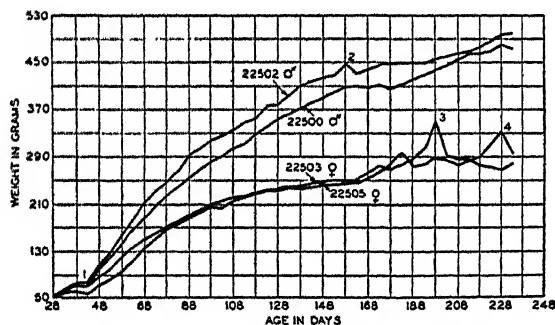


Fig. 7 Weight curves of individual rats receiving 160 γ of crystalline B per day. At point 1 the administration of the vitamin in 5-day portions was started. At point 2 no. 22502 was mated with 22503 and no. 22500 with 22505. At points 3 and 4, nos. 22503 and 22505 had litters of ten and nine young, respectively.

case. A more probable explanation is that large amounts of B exercise a growth acceleration sometimes confused with that due to B<sub>4</sub>.

These results raise several profound questions with regard to the physiological action of this vitamin. It is obvious that B is not solely an antineuritic vitamin but plays an important role in the general physiological economy. It should be noted that the upper limit of beneficial increment of the vitamin has not been reached although the vitamin intakes which can generally be secured by a choice of natural foods has apparently been surpassed. The effect of large B supplements to rich natural diets is being studied. Obviously, the experiments need to be extended to cover the life cycle and successive generations of animals to determine the ultimate effects of a wide range of B intake on longevity, size, reproductive functions, etc. Such experiments are now in progress.

#### SUMMARY

1. The various units of B assay have been compared by means of feeding tests with crystalline vitamin B.
2. The effects on growth of increasing doses of crystalline B, ranging from 0.5  $\gamma$  to 160  $\gamma$  per day, have been observed.
3. The stimulative effect persists up to and probably beyond a dosage of 160  $\gamma$ , an amount 80 to 160 times that necessary for maintenance of life.

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# FACTORS AFFECTING THE CAROTENE CONTENT OF ALFALFA HAY AND MEAL<sup>1</sup>

H. R. GUILBERT

*Division of Animal Husbandry, College of Agriculture, University of California,  
Davis*

ONE FIGURE

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Recognition of the importance of vitamin A in commercial livestock and poultry production has resulted in considerable work on the variation in vitamin A value of alfalfa hay and meal, the sources most commonly employed to supply this food essential. The literature, most of which has been reviewed recently by Scheunert and Schieblich ('34), is, however, somewhat confusing. The valuable contribution of Russell ('29), which showed that a sample of artificially dried alfalfa contained at least seven times as much vitamin A as a field-cured sample that had lost most of its green color, became widely quoted, and unjustifiable, broad generalizations were made on the basis of this figure. Dehydration plants were built and the products sold on the assumption that all samples of artificially dried alfalfa contained seven to ten times as much vitamin A as field-cured products. Although most subsequent tests have shown greater vitamin A potency in machine dried than in field-cured hays, the relative values have been variable and in at least two cases (Kiesselbach and Anderson, '31; Guilbert, '34) no significant difference has been found.

<sup>1</sup> This report is part of an investigation on the relation of nutrition to reproduction in livestock which became cooperative with the U. S. Bureau of Animal Industry, July 1, 1929.

In 1931, Hauge and Aitkenhead reported on the effect of artificial drying upon the vitamin A content of alfalfa. They stated in their discussion, "These experiments clearly demonstrate that the practice of treating alfalfa at elevated temperatures in mechanical dryers is without deleterious effect on vitamin A. Even a temperature as high as the hot flue gas used in one of the machines, or the sterilization temperature of the autoclave, was not destructive." They further stated, "that the sun's rays (ultra-violet rays) are not responsible for the destruction of vitamin A during the field curing process." These authors concluded also that enzymes are the important factor in the destruction of vitamin A during curing. Later experiments (Hauge, '34) gave essentially the same results. The implication that artificially dried alfalfa is the equivalent of fresh green forage does not appear to agree with observed results under commercial conditions nor does it agree with the work of Sherwood and Fraps ('32) and of Fraps and Treichler ('33). Scheunert and Schieblich ('34) also assumed that there was little or no loss of vitamin A in artificial drying, but no adequate means was employed to test the original material.

In a recent paper (Guilbert, '34) some of the most important work, justifying the use of carotene analyses as a means of determining vitamin A value of forage has been summarized. The accumulated evidence leads to the conclusion that the vitamin A problem in alfalfa is essentially a carotene problem. Carotene is well known to be readily oxidized and at least since the time of Capranica (1877) has been recognized as a light-sensitive compound. These facts alone would appear to cast some doubt upon the conclusions of Hauge and Aitkenhead ('31) which minimized the importance of sunlight and oxidation. Our own data showed extremely rapid destruction of carotene in dry alfalfa meal when exposed to sunlight (under glass) which could not be accounted for by enzyme action nor by ultra-violet rays. It was consequently decided to investigate further the factors affecting the carotene content of alfalfa hays and meals. The technic

used for the carotene determinations is described in a previous publication (Guilbert, '34).

#### EXPERIMENTAL

Smith and Milner ('34) have shown that practically all of the carotene in alfalfa leaves is the optically inactive beta form. The carotene values presented in this report are expressed, therefore, on the basis of beta carotene. The dye solution used in the colorimetric determinations was standardized against a sample of beta carotene kindly furnished to us by Dr. H. H. Strain, Division of Plant Biology, Carnegie Institution of Washington, Stanford University, California. We have previously reported carotene values of alfalfa upon the basis of International Standard carotene. The values reported here may be converted to the International Standard basis by multiplying by the factor 1.12.

Unless otherwise stated the samples of alfalfa were cut and brought immediately to the laboratory. Less than an hour elapsed between the time of cutting and the time the sample was either in process of analysis or undergoing the treatment specified in subsequent tables. Dried samples that could not be analyzed at once, were placed in stoppered tubes and stored at a temperature below 0°C., a condition shown to be adequate to prevent deterioration. All analyses are calculated to the moisture-free basis.

*Effect of vacuum-drying.* Four tests were run comparing the carotene analyses of fresh alfalfa leaves, and samples of the same leaves after vacuum-drying for 3 hours at 100°C. The carotene values obtained from the fresh leaf samples were 48.9, 52.0, 46.6 and 60.3 mg. per cent, respectively, while the values on the corresponding vacuum-dried samples were 48.4, 51.3, 46.6 and 57.1 mg. per cent. Thus there was little or no loss in vacuum-drying three of the four samples and the apparent loss in the fourth sample was 5.3 per cent. These results are in agreement with the well-known fact that carotene (and vitamin A as well) is relatively stable to heat in the absence of oxygen.



Since vacuum-drying is more convenient and assures more accurate sampling of the small amount required for carotene determinations than fresh leaves, all reference or control samples have been vacuum-dried for 3 hours at 100°C.

*Effect of autoclaving for 1 hour at 17 pounds pressure.* Hauge and Aitkenhead ('31) indicated that autoclaving did not diminish the vitamin A potency of alfalfa. The value of their original material, however, was not determined. Presumably no loss would occur in an autoclave from which the air could be rapidly evacuated. In our experiments a horizontal Bramhall Deane gas-heated autoclave was used, the inside dimensions of which were 26 × 36 inches. Two to 4 pounds of freshly cut alfalfa were used in each test. The time required for the temperature to approach the theoretical value for 17 pounds pressure was 15 to 20 minutes. In a series of four tests the vacuum-dried control samples contained 57.1, 57.4, 58.1 and 47.9 mg. per cent of carotene, respectively. The corresponding samples, autoclaved, then vacuum-dried contained 18.6, 25.0, 38.7 and 25.3 mg. per cent of carotene, the loss varying from 33 to 67.5 per cent. Tests on two of the samples showed that all of the chromogenic substances in the unsaponifiable fraction that react with antimony trichloride to form a blue color, decreased in proportion to the carotene.

*Effect of sun-exposure.* In table 1 are presented the results of several tests on the effect of sun-drying, and of sun-exposure on dried samples. Reference to test 1 in the table shows a loss of 69.5 per cent of the carotene in a sun-dried leaf sample taken from whole plants that were exposed in a layer approximating the thickness of hay in the swath. The leaves were practically dry in a few hours so that the period during which enzyme action might occur was relatively short. A vacuum-dried sample covered with ordinary glass and exposed to the sun at the same time decreased in carotene content almost as much as the sun-dried sample. The vacuum-dried sample, under glass, was subjected during most of the period of sun-exposure to a temperature averaging

about 65°C. or 15°C. higher than that of the sun-dried sample. To check the effect of the temperature difference, a portion of the control sample was subjected to a temperature 65° in the dark for 12 hours. This sample decreased in carotene content only 6.8 per cent. Since there was little difference in the rate of destruction in the sun-dried sample and the

TABLE 1  
*Effect of sun-exposure on the carotene content of alfalfa leaves*

TEST NO.	TREATMENT	CAROTENE, MILLIGRAMS PER 100 GM. (DRY BASIS)	PERCENTAGE LOSS
1	Leaves, vacuum-dried (control)	57.1	
	Leaves, sun-dried. Twelve hours sun-exposure, taken into laboratory 28 hours after cutting	17.4	69.5
	Leaves, vacuum-dried then exposed to sunlight the same as above, except that it was under glass	18.6	67.4
	Leaves, vacuum-dried, then exposed for 12 hours in the dark to a temperature of 65° in the presence of air	53.2	6.8
2	Leaves, autoclaved then vacuum-dried (control)	18.6	
	Leaves, autoclaved then sun-dried the same as in test 1	8.6	53.8
3	Leaves, vacuum-dried (control)	58.1	
	Leaves, sun-dried 12 hours taken to laboratory 27 hours after cutting	21.1	63.7
4	Leaves, autoclaved then vacuum-dried (control)	38.7	
	Leaves, autoclaved then sun-dried as in test 3	26.3	32.0
5	Leaves, vacuum-dried (control)	47.9	
	Leaves, sun-dried, 6½ hours semi-cloudy, 7½ hours bright sunlight, taken to laboratory 28 hours after cutting	25.7	46.4
6	Leaves, autoclaved then vacuum-dried (control)	25.3	
	Leaves, autoclaved then sun-dried as in test 5	18.6	26.5
7	Dehydrated alfalfa leaf meal (control)	12.3	
	Sample after 8 hours sun-exposure under glass	6.0	51.2
	Sample after 16 hours sun-exposure under glass	3.6	70.7
	Sample after 40 hours sun-exposure under glass	2.1	83.3

previously dried sample when exposed to the sun during the same period of time, it appears that, under conditions favorable for rapid drying, temperature and enzyme action were minor factors compared to the photo-chemical activation of the destructive process. Furthermore, the sample shown in test 2 which was autoclaved to stop enzyme action, suffered a loss of 53.8 per cent of its carotene when exposed to the sun simultaneously with the samples of test 1.

Tests 3, 4, 5, 6 and 7 in table 1 offer additional data on the loss from sun-drying and from sun-exposure of previously dried samples. These data show that autoclaving to prevent enzyme action reduced, but by no means stopped the destruction of carotene during sun-drying, and that contrary to the conclusions of Hauge and Aitkenhead ('31) the sun's rays play an important part in the destruction of vitamin A potency during field curing. That ultra-violet rays are not necessarily involved, is shown by the fact that destruction of carotene proceeds rapidly in samples covered with ordinary glass. In tests 3, 4, 5 and 6 the rate of sun-drying was slower than in tests 1 and 2 and the apparent loss from enzyme action (the difference in percentage loss between fresh and autoclaved samples) was greater.

*Effect of enzyme action.* Further study of the effect of enzyme action on the carotene content of alfalfa leaves is presented in table 2. In the dark, at room temperature (20° to 30°C.), some process in the living plant tissue caused a loss of 8.5 per cent of the carotene in 24 hours and 54.1 per cent in 75 hours. A sample from hay exposed over-night in a windrow and to sunlight in the late evening and early morning for about 4 hours, showed a loss of 10.2 per cent of its carotene.

A sample of leaves was placed over water to which was added a small quantity of toluene and kept in an incubator room at a temperature of 38°C. The air in the incubator was practically saturated with moisture. These conditions are somewhat similar to those in the experiments of Hauge and Aitkenhead ('31). At the end of 24 hours the alfalfa

leaves had the dark olive-drab color of autoclaved samples and 95 per cent of the carotene had been lost. There was no evidence of mould, but it appears doubtful that this technic would be effective in eliminating bacterial action. An autoclaved sample under the same conditions lost 33 per cent

TABLE 2

*Effect of enzyme action on the carotene content of alfalfa leaves*

TEST NO.	TREATMENT	CAROTENE, MILLIGRAMS PER 100 GM. (DRY BASIS)	PERCENTAGE LOSS
9	Leaves, vacuum-dried (control)	57.1	
	Leaves, after standing in dark at 20 to 30°C. for 24 hours without wilting, then vacuum- dried	52.3	8.5
	Leaves, after standing in dark at 20 to 30°C. for 75 hours without appreciable wilting, then vacuum-dried	26.2	54.1
	Leaves, after standing from 5 P.M. to 8 A.M. in the field in a windrow then vacuum-dried	51.3	10.2
	Leaves, placed over water and toluene for 24 hours at 38°C., then vacuum-dried	2.9	94.9
10	Leaves, autoclaved then vacuum-dried (control)	18.6	
	Leaves, autoclaved then placed over water and toluene for 24 hours at 38°C., then vacuum- dried	12.4	33.3
11	Leaves, vacuum-dried (control)	47.9	
	Leaves, exposed at 38°C. over water and toluene for 24 hours, then vacuum-dried	24.5	48.9
12	Leaves, autoclaved then vacuum-dried (control)	25.3	
	Leaves, autoclaved then incubated and vacuum- dried in test 11	25.3	0.0

of its carotene (test 10). It appears, therefore, that enzyme and possibly bacterial action was important in the rapid destruction of carotene in the incubated fresh leaf sample of test 9.

The results of a second trial are shown by tests 11 and 12 in table 2. In this experiment a sample of fresh leaves and a sample of autoclaved leaves were placed in open desiccators

over water and toluene and were kept in an incubator at 38°C. for 24 hours. The air in this incubator was relatively dry, causing slight wilting of the fresh leaf sample during the night. The autoclaved sample remained wet throughout the 24-hour period. The fresh leaf sample, which remained green in color, lost 49 per cent of its carotene under these conditions while there was no loss in the autoclaved sample.

TABLE 3  
*Effect of temperature on the loss of carotene in alfalfa during storage*

TEST NO.	TREATMENT	CAROTENE, MILLIGRAMS PER 100 GM. (DEY BASIS)	PERCENTAGE LOSS
13	Leaves, sun-dried (control)	17.4	...
	Sample after 8 weeks storage at 0 to 5°C.	17.9	
14	Leaves, vacuum-dried (control)	57.1	...
	Sample after 8 weeks storage at 0 to 5°C.	56.9	
15	Sun-cured alfalfa meal (control)	6.6	...
	Sample after 8 weeks storage at —5 to 0°C.	6.7	
16	Dehydrated alfalfa meal (control)	13.6	...
	Sample after 8 weeks storage at —5 to 0°C.	13.2	
17	Sun-cured alfalfa meal (control)	7.9	30.4
	Sample after 8 weeks storage at 20 to 30°C.	5.5	
18	Dehydrated alfalfa meal (control)	16.7	30.5
	Sample after 8 weeks storage at 20 to 30°C.	11.6	
19	Fresh leaves, vacuum-dried (control)	55.4	61.9
	Sample after 9 days in the dark at 60°C.	21.1	
	Sample after 9 days in the dark at 80°C.	7.3	

These data indicate that during warm cloudy weather, or when moist hay heats in the cock or stack, loss of carotene from enzyme action may be rapid. Bacterial action under these conditions should also be considered.

*Effect of environmental temperature on the loss of carotene during storage.* Fraps ('33) and Sherwood and Fraps ('32) have demonstrated by biological tests, that the vitamin A potency of natural feeds deteriorates during storage.

Russell, Taylor and Chichester ('34) found that there was no loss of carotene in alfalfa samples stored in vacuo at  $0^{\circ} \pm 5^{\circ}$ . These authors also indicate that the rate of loss of carotene in dried plant tissue may be greater during the first few months of storage than after a longer period, while data from Fraps and Treichler ('33) indicate the opposite.

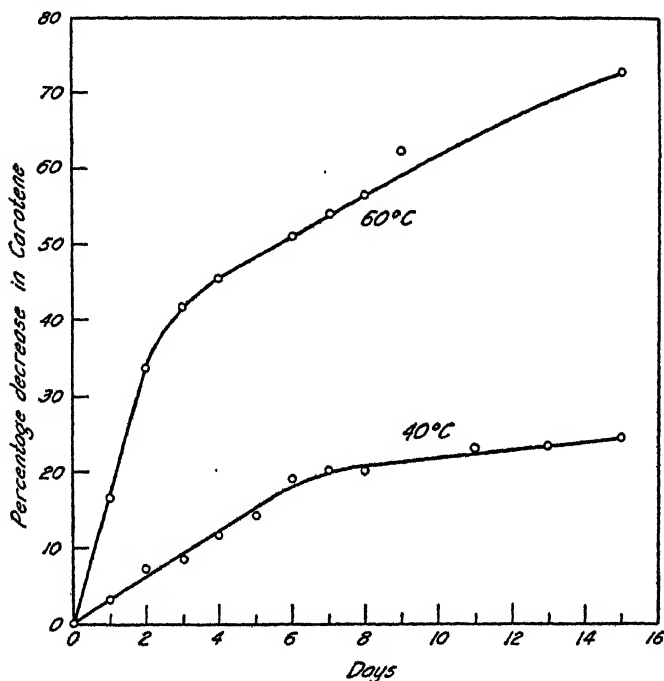


Fig. 1 Percentage decrease in carotene content of vacuum-dried alfalfa leaves stored in the dark at temperatures of  $40^{\circ}$  and  $60^{\circ}\text{C}.$ , respectively.

Data on the destruction of carotene in alfalfa at different temperatures are presented in table 3. There was no loss in samples either in stoppered tubes, or in heavy paper sample containers, when stored for 8 weeks in the dark at temperatures of  $-5^{\circ}$  to  $5^{\circ}\text{C}.$  (tests 13, 14, 15 and 16). There was a 30 per cent loss of carotene in alfalfa meals stored in the dark, in paper sample containers, for a period of 8 weeks at room temperature (about  $20^{\circ}$  to  $30^{\circ}\text{C}.$ ) (tests 17 and 18).

Test 19 shows the effect of exposure in the dark for 9 days at temperatures of 60° and 80°C., respectively. Oxidation undoubtedly being the cause of the carotene loss, acceleration of the rate at higher temperatures, is to be expected.

The percentage decrease in carotene content of finely ground alfalfa leaves placed in ovens at 40°C. and 60°C., respectively, during a period of 15 days is presented graphically in figure 1. The rate of destruction at comparable phases of the curves was roughly doubled for each 10° rise in temperature. There was only a slightly smaller loss in whole leaves than in finely powdered leaves over the 15-day period. At both 40°C. and 60°C. there was an initial phase during which the rate of destruction was more rapid than it was subsequently. The inflection of the curve at 60°C. came at 3 days while at 40°C. it came on the sixth or seventh day. At the lower temperatures obtaining under average conditions of storage this initial phase might be prolonged for a considerable time and may partially account for the idea expressed by Russell, Taylor and Chichester ('34) that the rate of loss of carotene may be greater during the first few months of storage than after longer periods.

*Deterioration in carotene content of alfalfa under different conditions of storage.* Samples were obtained at approximately monthly intervals from 5-ton test lots of alfalfa meal, stored in a galvanized iron warehouse located in the northern part of the Sacramento Valley. The meal was packed in burlap bags containing 100 pounds each. The results of the carotene analyses together with the mean monthly temperatures are shown in table 4. The temperature record was obtained from a U. S. Weather Bureau station located 10 miles distant, but at about the same elevation and the temperatures of the two places are similar. No record on the temperature inside the warehouse was available, but it was stated that the temperature was not uncommonly 10°F. lower than outside air temperature. The temperature of the alfalfa meal after dehydration, grinding and packing into 100 pound bags was usually about 100°F. Hence, it is reasonable to

assume that the mean temperature of the meal during the period of storage investigated was 70°F. or higher.

While some inconsistencies appear in the results, which are probably caused by error in sampling, serious losses unquestionably occur under these conditions. Dehydrated meal after several months of storage may be much lower in carotene than freshly prepared sun-dried meal, carefully cured under favorable conditions. The rate of loss declined during the cool weather in October and November.

TABLE 4

*Decrease of carotene content of alfalfa meal during storage in a warehouse from June 26 to December 6, 1934*

DATE	CAROTENE, MILLIGRAMS PER 100 GM.					MONTHLY TEMPERATURE U. S. WEATHER BUREAU	
	Lot 1 Dehy- drated	Lot 2 Dehy- drated	Lot 2 Sun- cured	Lot 3 Dehy- drated	Lot 3 Sun- cured	Mean	Highest
June 26	11.3	11.4	7.9			°F. June 76	°F. 113
July 12	...	...	...	16.7	7.9		
July 27	8.9	6.9	7.1	...	...	July 81.2	109
Aug. 8	...	...	...	13.2	6.6		
Aug. 27	6.3	5.9	7.0	...	...	Aug. 82.3	108
Oct. 1	...	4.6	4.1	...	...	Sept. 77.3	107
Oct. 9	7.1	...	...	10.3	5.1	Oct. 65.0	100
Nov. 7	5.8	4.5	3.9	6.5	4.8	Nov. 54.2	77
Dec. 6	5.7	3.9	4.9	6.7	4.8		

Samples were taken from a lot of chopped, sun-cured hay that was stored in a dark loft at the University Farm, Davis. The values obtained in November, 1933, April, 1934 and August, 1934 were 3.3, 3.0 and 2.1 mg. per cent of carotene, respectively. During the comparatively cool period from November to April the decrease was about 9 per cent, while during the hot weather from April to August the decrease was 30 per cent.

While other factors than temperature may be involved in changing rates of deterioration, as previously indicated, it appears probable that temperature variations may explain the discordant results previously reported. Thus if meal goes



into storage in the spring, comparatively rapid loss may be expected during the summer months and little loss during the succeeding fall and winter. If, however, the alfalfa is stored from late summer or fall crops, little loss might be expected during the following cold months and heavy losses during hot weather of the next spring and summer.

*Seasonal variation in carotene content of dehydrated and sun-cured alfalfa meals.* A series of samples was obtained from a ranch in the Sacramento Valley that produces both sun-cured and dehydrated alfalfa meals. The alfalfa all came from the same ranch and the sun-cured and dehydrated lots from which the test samples were taken were in each case cut at the same time and from adjoining checks in the same field. Because the capacity of the dehydrator was greatly increased by a 10 to 15 per cent reduction of moisture in the field, and because of the practical difficulties of supplying hay to the dehydrator immediately after cutting, the hay had more or less exposure in the field before dehydration. In some cases the hay was cut, raked within an hour and hauled to the dehydrator a few hours after cutting. In other instances the hay was cut in the late afternoon, raked into windrows and left in the field over-night, to have a supply ready for the dehydrator in the morning. The dehydrator employs, in the process, flue gases along with considerable excess air. The chopped hay is first blown into the hot gas stream at the base of a verticle, funnel-shaped tower where it is floated out of the top as moisture is lost. The hay remains in this part of the dehydrator a maximum of 30 seconds where the temperature of the flue gases at the base ranges from 900° to 1200°F. depending on the moisture content of the hay. The drop in temperature is extremely rapid. Next it goes through a revolving, horizontal drum at a temperature varying from 230° to 270°F. The whole process requires about 15 to 18 minutes. The leaves and finer particles, however, go through the dehydrator in 2 to 3 minutes.

In this area the relatively low humidity and high temperatures throughout the summer are favorable for rapid sun-curing. The hay was carefully handled to facilitate drying

and to minimize sun-bleaching. The period elapsing between the time of cutting and milling varied from 2 to 5 days. Usually it was ground and sacked on the fourth day. A typical example of the sun-curing process is as follows: "The hay was cut, raked into windrows and cocked on July 19. The cocks were turned July 21, loaded on trucks the evening of July 22 and milled the morning of July 23."

The hay, throughout the season, was cut after 3 to 4 weeks of growth and the samples were received and analyzed within a week after curing. The data are presented in table 5.

TABLE 5  
*Seasonal variation in dehydrated and sun-cured alfalfa meals*

DATE OF CUTTING	DEHYDRATED ALFALFA MEAL, CAROTENE MILLIGRAMS PER 100 GM.	SUN-CURED ALFALFA MEAL, CAROTENE MILLIGRAMS PER 100 GM.
May 24, 1934	11.3	...
June 21, 1934	11.4	7.8
July 5, 1934	16.7	7.9
July 19, 1934	13.7	8.2
August 9, 1934	15.7	8.5
August 23, 1934	18.4	7.3
September 13, 1934	20.9	12.1
October 3, 1934	22.2	12.1
October 29, 1934	29.6	...

In both the sun-cured meals and the dehydrated, but in the latter particularly, there was a marked trend toward higher carotene values from spring to autumn. The variables that may have a bearing on these results are so numerous that definite interpretation is impossible. There is good evidence, however, that considerable loss occurred between the time of cutting and dehydration. This loss would depend on temperature, enzyme action, and intensity of sun-exposure during the variable time elapsing between cutting and dehydrating. Decrease in loss before dehydrating caused by cooler weather and less intense sunlight may have contributed significantly to the higher values obtained in the fall. Improvements in field handling that tended to minimize exposure

during periods of most intense sunlight were also initiated during the latter part of the season. Furthermore, differences caused by variation in the stage of maturity of the plants must be considered. The last cutting, for example, because of slower growth, was more immature when harvested than earlier cuttings. The following data are pertinent to the question of the influence of stage of maturity upon the carotene content of the plants.

Analyses of vacuum-dried stems and leaves at various stages of growth have given carotene values for the stems varying from 6 to 11 mg. per cent while the leaves varied from 45 to 60 mg. per cent. Data collected by the Division of Agronomy, University of California, on three varieties of alfalfa (Madson, '31) shows that the growth 15 days after harvesting consisted of about 60 per cent leaves and 40 per cent stems. At 22 days the proportion was about equal and at 30 days' growth the proportion was 55 per cent stems and 45 per cent leaves. It appears, therefore, that proportion of stems to leaves, hence stage of maturity, would play a dominant role in determining the carotene content of the plant. Hauge ('34) found the highest vitamin A value in young alfalfa 10 to 12 inches high and he further found by biological tests that leaves were ten to fourteen times as potent as the stems. The highest values for vacuum-dried whole plants, which we have thus far obtained, have been from immature plants 10 to 12 inches high cut early in November. The values both from the fields from which the October 29th sample in table 5 came, and from similar growth on the University Farm were from 39.4 to 42.7 mg. per cent. The data on stage of growth and possible seasonal variation in relation to carotene content are yet too meager to warrant conclusions. That the carotene content of the original plant material is one of the factors influencing the variation in the carotene content of dehydrated meals shown in table 5, is, however, strongly indicated.

*Evidence of loss during mechanical dehydration.* Most of the fresh samples of dehydrated alfalfa meals that we have analyzed contained from 13 to 22 mg. per cent of carotene.

The highest values reported by Russell, Taylor and Chichester ('34) were from 18 to 22 mg. per cent. The values obtained for vacuum-dried whole plants cut at various stages of growth have varied from 27 to 42 mg. per cent. This circumstantial evidence would lead one to the conclusion that serious losses must occur during mechanical dehydration. In some instances losses of carotene between the time of cutting and dehydration and also storage losses must be considered.

Russell, Taylor and Chichester ('34) found no loss in dehydration, in fact they obtained slightly lower carotene values on the fresh samples of alfalfa than for the dehydrated. They state, however, that they had some difficulty in getting the fresh material into a finely divided state for extraction and that some loss may have occurred between the time of harvesting and analysis. This time was not stated except that the samples were analyzed 'within a week.'

Direct evidence regarding loss in dehydration was obtained from one series of samples secured from the ranch previously mentioned, the data from which are shown in the following tabulation:

	<i>Carotene milligrams per 100 gm.</i>
Sample at time of cutting, 3 P.M., October 25	41.3
Sample when delivered at dehydrator, 10.30 A.M., October 26, after standing in windrow over-night	36.6
Sample of dehydrated meal	33.9

The samples taken at the time of cutting and on delivery at the dehydrator were placed in sealed mason jars and kept at 0°C. until delivered at the laboratory 24 hours after cutting. They were then immediately vacuum-dried and stored in the cold until analyzed. The percentage loss in the field in this test was 11.4 per cent and the loss in the dehydrator was 7.4 per cent. The total loss in these operations was 18 per cent of the original carotene.

The results of one test should not be considered conclusive because of possible errors in sampling. The data indicate, however, that losses occur in mechanical dehydration. Incidentally the value of the meal in this test (33.9 mg. per cent) is the highest we have encountered.

## DISCUSSION

Factors influencing the carotene content of alfalfa hays and meals have been studied by means of carotene determinations, using a simplified technic for routine analysis described in a previous publication (Guilbert, '34).

Little or no loss occurred in vacuum-drying, a finding in agreement with the well-known fact that carotene is relatively stable to heat in the absence of air.

Autoclaving resulted in significant losses of carotene presumably because the air could not be rapidly exhausted from the chamber of the autoclave used in these experiments. Losses apparently occurred also in the experiments of Hauge ('34), as the highest value reported for leaves that were autoclaved and then sun-dried was 140 Sherman units per gram. On the basis of present standards this value would be equivalent to approximately 12 mg. of carotene per 100 gm.—a comparatively low value for alfalfa leaves.

Extremely rapid loss of carotene results from sun-drying or from exposing dried alfalfa to either direct sunlight or sunlight filtered through ordinary glass. Autoclaving to stop enzyme action reduced but by no means eliminated loss of carotene during subsequent sun-drying.

Enzyme action was found to be an important factor in loss of carotene during the curing process. Under favorable conditions for sun-drying, however, enzyme action and temperature appeared to be minor factors compared with photo-chemical activation of the destructive process. Under unfavorable conditions for drying, prolonged enzyme action coupled with other destructive processes may result in hay with very low carotene content.

Temperature was shown to be the major factor causing variation in loss of carotene in alfalfa hay or meal during storage. No loss occurred over a period of 8 weeks at  $-5^{\circ}$  to  $5^{\circ}\text{C}$ . while losses of 30 per cent occurred during a similar time at  $20^{\circ}$  to  $30^{\circ}\text{C}$ . The loss during 9 days at  $60^{\circ}\text{C}$ . and  $80^{\circ}\text{C}$ . was 62 and 87 per cent, respectively. Thus the rate of loss increases as the temperature rises, tests over 15 days

at 40° and 60°C., respectively, indicating that the rate of loss is roughly doubled for each 10° rise in temperature. Large losses of carotene occurred in dehydrated and sun-cured meals stored in a warehouse during hot weather. After 3 or 4 months dehydrated meals may be significantly lower in carotene than freshly prepared sun-dried meal, carefully cured under favorable conditions.

The carotene content of dehydrated alfalfa meals analyzed in this laboratory and also that reported by others are markedly lower in carotene than samples of whole plants cut at various stages of growth and vacuum-dried. This difference may be accounted for by losses in the hay before delivery to the dehydrator, losses during dehydration and by losses during storage.

#### CONCLUSIONS

The accumulated evidence summarized in a previous publication (Guilbert, '34) leads to the conclusion that the vitamin A problem in alfalfa is essentially a carotene problem.

The variability in carotene content of alfalfa hays and meals, both dehydrated and sun-cured, is so great, not only because of destruction during curing and storage, but also because of variation in the original hay as affected by stage of maturity, etc., that no sweeping generalities should be made regarding the relative values depending on method of curing.

Recognition of the various factors affecting the carotene content of alfalfa hay and meal should make possible a reasonable degree of standardization of products prepared especially for their vitamin A value. Such standardization would necessarily be subject to time limitations, however, unless effective means are employed to prevent deterioration in storage.

Feed dealers and cooperatives who are buying large quantities of alfalfa meals to incorporate into rations primarily as a source of vitamin A would do well to consider purchasing on the basis of carotene content, since the analytical technic is no more complicated, nor expensive than the other feed analyses commonly made.

#### ACKNOWLEDGMENT

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# THE EFFECT OF RETARDED GROWTH UPON THE LENGTH OF LIFE SPAN AND UPON THE ULTIMATE BODY SIZE <sup>1</sup>

C. M. McCAY, MARY F. CROWELL AND L. A. MAYNARD  
*Animal Nutrition Laboratory, Cornell University, Ithaca*

## ONE FIGURE

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In a preliminary report, the literature concerning the effect of retarded growth upon the life span was reviewed (McCay and Crowell, '34). In this report was also included a summary in the nature of a progress report dealing with a study employing rats to determine the effect of retarding growth upon the total length of life.

The present summary represents a complete, final report of this experiment employing white rats and covering a period of nearly 4 years. The object of this study was to determine the effect of retarding growth upon the total length of life and to measure the effects of retarded growth upon the ultimate size of the animal's body. In the present study, growth was retarded by limiting the calories.

The growth of an animal can be retarded either by disease or by various nutritional deficiencies. Every laboratory that performs vitamin assays is familiar with the retarded growth and prompt death that result when there is a deficiency of a certain factor such as vitamin A. In such experiments the animal grows little and dies prematurely. However, a borderline level of such an essential as vitamin A may permit a very slow growth and permit the animal to approach or attain

<sup>1</sup> These studies were supported in part by the Snyder research grants and we appreciate the assistance of Mrs. Harry Snyder in making these studies possible.



adult size. Under such conditions the question arises as to the effect of this retarded growth upon the life span and ultimate body size. In the present study we have retarded the growth by restriction of calories, but with a diet designed to provide adequate levels of all other constituents.

In our previous report some of the earlier literature dealing with the interrelationship between the rate of growth and the length of life, was reviewed. The existence of such a relationship has been stressed again very recently by Kermack and associates ('34) in the following words:

It is shown that these results are consistent with the hypothesis that the important factor from the point of view of the health of the individual during his whole life is his environment up to the age of say 15 years and that improved conditions at later ages have little direct effect.

This is a recognition that factors during the growing period are paramount in their influence upon the subsequent life span.

A few attempts have been made in other laboratories to test the hypothesis that a slow rate of growth results in an increased life span, by feeding a given species, such as mice, the same diet, recording the rate of growth and finally the length of life. At the conclusion of such an experiment the rates of growth and the length of life have been correlated. On the basis of such a study Robertson and Ray ('20) concluded that mice that grew the more rapidly lived the longer. They also concluded that the group that grew the slower was more unstable. Sherman and Campbell ('34) report, however, that rate of growth and length of life of rats on the same diet, vary independently of each other. Earlier, Campbell ('28) found that an improvement in the diet resulted both in increased rate of growth and increased length of life in the same individuals.

It is doubtful if such studies as those of Robertson really test the hypothesis, because the two groups, separated on the basis of growth, are not homogeneous. The slower growing group tends to include the inferior individuals that die prematurely. A genuine test of this hypothesis can be made,

however, if animals are separated into groups at the time of weaning or shortly thereafter. Such groups can be forced to grow at different rates and the length of life can be determined. In this latter case we are dealing with more homogeneous samples. Random selection provides reasonable insurance of similar expectancies of life spans and rates of growth in such groups, if other factors are constant.

#### EXPERIMENTAL

At the present time it seems that the only method of determining the interrelationship between the rate of attaining maturity and the total length of life is by direct experiments upon homogeneous groups of animals. For this reason 106 white rats were divided into three groups at the time of weaning. One group contained thirty-four individuals and the other two thirty-six. Group I was allowed all the feed desired and grew normally. Group II was restricted in feed intake from the time of weaning. Group III was allowed sufficient feed to permit normal growth for 2 weeks after weaning and then restricted in the same manner as group II.

These animals were confined in false-bottom cages such as those used for vitamin assays except that individuals tended to develop sore feet from time to time as old age approached. In these cases they were placed upon a solid bottom covered with shavings until the feet healed.

A diet was desired that would provide an excess of all recognized essentials for rapid growth except sufficient calories.

The diet designed for these experiments was a synthetic mixture of starch 22, cellulose 2, lard 10, sucrose 10, salt mixture 6, dried yeast 5, cod liver oil 5 and casein 40. The starch was cooked and dried. The cellulose was the regenerated product described elsewhere (McCay, '34). The salt mixture was that of Osborne and Mendel. The casein was not purified.

This diet was made high in all dietary essentials in order that the retarded-growth rats with restricted daily intakes

might have adequate protein, minerals and vitamins. In order to further compensate for the possible shortage of vitamins in the retarded growth groups, an additional 3 drops of cod liver oil and 0.5 gm. of yeast were fed daily to each member of groups II and III. This was estimated to compensate for the vitamins in the larger amounts of diet ingested by the rapidly growing group, no. I.

In the use of this diet which is rich in essentials such as protein, it is recognized that the group ingesting food *ad libitum*, may be subject to injury by the excess above the requirements of the body. It is not likely that such injury was produced in the present case, however, since the animals that matured rapidly had life spans similar to those found previously in our colony upon stock diets.

In a study of retarded growth the animal can be retarded for a long period and then allowed to grow. Osborne and Mendel ('15) employed this method. An optional procedure consists in holding the animal at a constant weight for a period of weeks, then allowing it to make a slight gain at a normal growth rate and following this in turn by another period of constant body weight. This procedure was used in an earlier study with brook trout (McCay, Dilley and Crowell, '29). This 'stairstep' method was employed in the present study with rats.

The feed for each animal of the retarded growth groups was weighed separately each day. The individuals were weighed three times weekly. The allowance of feed was thus adjusted to hold the body weight of each member of the retarded groups as nearly constant as possible. Usually a growth of 10 gm. was permitted to each individual of the retarded groups at intervals of 2 to 3 months. The period of holding them at a constant weight was determined by the appearance of the animals. As soon as any members of the retarded groups seemed to be failing from the deficiency of calories the entire group was allowed to grow to the extent of the 10 gm.

Two different methods were employed during the period of 10 gm. growth. Part of the time an allowance of sucrose was given in addition to the usual maintenance allowance of feed. Growth became normal in every case showing that the diet was adequate except for calories. In most cases, however, fresh beef liver was fed during this growth period. An equal allowance of this liver was given the animals of group I.

At the time the surviving rats were 766 days old, the retarded-growth groups were each subdivided. Half of each group was given all the feed it desired. The other half in each case was continued on the restricted intake until the 911th day. From that period all animals were allowed all the diet desired. The purpose of this subdivision was to determine if any difference in the power to resume growth existed between the 766th and the 911th days. Osborne and Mendel ('15), in the case of one rat, found growth was resumed after retardation for 552 days. After this retardation the rat attained a weight of 204 gm. and they stated that this was 'full size' for the female of this species.

In figure 1 are plotted the growth curves for the three groups. The curves at the top for group I show the rates for males and females separately. In the case of the retarded-growth animals the growth rates of the two sexes were maintained the same until the time for the resumption of growth. The ends of these curves show clearly that the male rat grows more rapidly and attains a larger size than the female even after growth has been retarded for more than 900 days. The power to grow still exists in the rat body after this period of 900 days.

At the top of figure 1 are shown the number of each sex alive in each group at various times. It will be observed that about forty rats were alive at the time of the first division on the 727th day.

These growth curves indicate that the retarded-growth rats attained about the same final weight as those of Osborne and Mendel, namely, 200 gm., but by comparison with the growth

curves for group I it is evident that the 'normal' size was not reached by the animals of the retarded-growth groups.

In table 1 are summarized the data regarding the weights attained which indicate the final size of the animal in terms of body weights.

In group I only animals that reached an age of 177 days or more are included. In groups II and III only those that

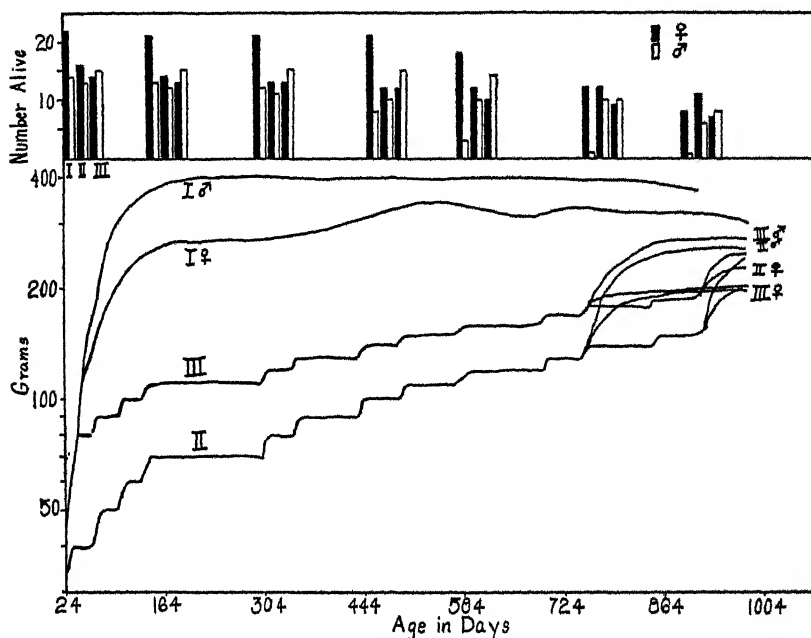


Fig. 1 Growth curves for rats allowed to grow rapidly, no. I, and for those retarded in growth by limiting the calories, nos. II and III. The columns at the top show the numbers alive by sex and group at various periods of the experiment. The top of these columns provides a mortality curve.

survived to make the final growth with ad libitum feeding are considered. These data are striking in the uniformity of the final weights of the retarded animals. The males of these retarded animals are significantly heavier than the females. Thus the undeveloped male of the species retains its potential growth power at a higher level than the female even after the male has lived for a period equal to nearly twice the average life span for this sex.

In terms of weight the difference between the final size of the animals that grew normally is significantly higher than those of the retarded groups. It is evident that animals retarded in growth for the long period that we have used are unable to attain the same final weight as those that grew rapidly to maturity. The tendency of white rats to attain a greater body weight than they did 20 years ago has been

TABLE 1

*Maximum weights in grams attained by groups and sub-groups. 'G' signifies the half of the group allowed to finish growth after 766 days and 'R' after 911 days*

GROUP	MEAN MAXIMUM WEIGHT ATTAINED	MEAN AGE OF ATTAINING MAXIMUM WEIGHT	MAXIMUM WEIGHT ATTAINED BY ANY INDIVIDUAL	NUMBER OF ANIMALS CONSIDERED
	<i>gm.</i>	<i>Days</i>	<i>gm.</i>	
I ♂	439±10	359±25	542	12
I ♀	355± 8	588±17	474	21
II ♂	262±13	1005±25	352	7
II ♀	210± 5	951±12	244	13
III ♂	267±12	903±17	358	9
III ♀	227± 5	918±26	260	10
IIG ♂	264±22	998±40	352	4
IIR ♂	259±17	1013±31	288	3
IIG ♀	223± 4	936±21	244	7
IIR ♀	195± 7	968± 8	233	6
IIIG ♂	266±20	851±18	358	5
IIIR ♂	268±13	968± 6	316	4
IIIG ♀	212± 5	840±18	238	6
IIIR ♀	256± 3	1036±27	260	4

observed in such laboratories as those at Yale. This suggests that there may be a close relation between the early growth rate and the final size since it is well recognized that the better knowledge of nutrition and selection have made it possible to stimulate growth and final size beyond the rates attainable 20 years ago. Our data suggest that a body of a certain size can be attained after very long periods of retarded growth, but that the attainment of the maximum size possible for a given species is impossible if growth is not permitted during early life. This is shown in table 1

by the attainment of the same maximum weights by groups II and III but both sexes of IIIg, the half of the group allowed to grow at 766 days, attained the maximum weights more quickly since they were larger when growth was permitted. They did not grow heavier, however, than those of group II.

The length of life for each individual of each group is given in table 2. The greatest length attained by any individual was 1421 days. There were members of each of the retarded-growth groups, nos. II and III, alive after all the members of group I had died. These data indicate clearly that some factor tended to promote longevity in the case of groups II and III. This effect is much more marked in the male sex than in the female. In two preceding reports (McCay and Crowell, '34; McCay, '34) it has been noted that the male rats of our colony tend to live an average of about 500 days.

The average life span of the male rats in the retarded-growth groups was 820 days in one case and 894 in the other. Upon the best diet among five, Slonaker's ('31) male rats averaged 767 days at the time of death while those of Campbell ('29) averaged 635 days upon her best diet. No male rat in Slonaker's recent experiments attained an age of 1200 days while one male among each six members of this sex of our retarded-growth groups exceeded this age. The males of our retarded groups tended to exceed the females in length of life.

The average life span of the females of the three groups is probably the same. No marked extension results from retarding the growth of this sex as far as our data indicate. Our data are somewhat distorted, however, due to the loss of two members of group II very early in the experiment. These were lost during a period of extremely hot weather. The best females in Slonaker's experiments lived an average of 848 days while the better of our retarded female groups averaged only 826 days. On the other hand, no female animal exceeded an age of 1250 days in Slonaker's study while four

of our retarded females, or about 10 per cent, exceeded this age. The average age of Miss Campbell's female rats on her better diet was 664 days. It is difficult to provide an explanation for this span of Campbell's animals which is so much shorter than either those of our own colony or those of Slonaker. Before studying the data of Slonaker it seemed

TABLE 2  
*Life span of individuals in days*

GROUP					
I ♂	I ♀	II ♂	II ♀	III ♂	III ♀
71	74	41	48	548	134
162	485	249	62	580	341
338	520	313	259	602	479
354	559	758	260	650	565
397	699	758	260	723	577
401	713	780	296	832	641
499	719	797	342	845	663
544	722	964	410	919	810
561	724	1006	755	978	822
563	733	1137	887	998	894
588	817	1244	901	1022	901
627	824	1294	904	1028	920
733	855	1321	925	1168	999
927	857		938	1218	1080
	870		980	1306	1111
	984		999		1123
	997		1041		1132
	1023		1107		1210
	1039		1225		1297
	1074		1232		
	1143		1261		
	1189		1304		
			1421		
483	801	820	775	894	826

that reproduction might have shortened the span of Miss Campbell's females, but this is hardly tenable when we consider that Slonaker's females also reproduced.

Since half of each retarded group of rats was allowed to complete its growth after 766 days and the remaining half retarded until after 911 days, it was thought that some difference in life span might result. The number of animals



alive at the time final growth was permitted was too small. The great variability in life spans of this limited number of cases made it impossible to find significant differences when these data were subjected to statistical treatment.

### *Hair growth*

Hair samples were clipped from the backs of rats on the 440th and 746th days of the experiment. The diameters of ten hairs from each rat were measured under the microscope with three readings at different places near the center of the hair. The diameters of these hairs are included in table 3. The hair of the female is always finer than that of the male even if the animals are maintained at the same body weight. At 460 days of age when group III animals weighed 140 gm. and those of group II weighed 100 gm., the hair of all retarded-growth animals was finer than that of the members of group I that were mature in size. At 746 days when group III had attained a mean weight of 170 gm. the hair had become as coarse as that of group I. The hair of group I had changed little if any. The hair of group II, the average weight of each member being 130 gm., had grown coarser but was still finer than that of the other groups.

When correlated with the body weight and age, these data indicate that hair grows coarser with both the increase in size and the increase in age of the individual. The hair changes may indicate that the rat is losing the qualities that characterize youth including the power to grow to a maximum size. It will be recalled from table 1 that our rats retarded for more than 700 days could not attain full adult size, while Osborne and Mendel claimed their rats could attain normal size after more than 500 days of retardation.

### *Organ weights*

At the time of death the rats were dissected and the weights of the heart, liver, spleen and kidneys were recorded. The blood was pressed from the heart before weighing. Only these data for animals that lived to grow without restriction

are included in table 3. These are the same animals whose maximum weights are recorded in table 1. No animals are included if they died before the opportunity to grow was provided. The number of animals upon which these organ

TABLE 3

*The effect of retarded growth upon the size of organs, hair and bones*

	GROUP					
	I ♂	I ♀	II ♂	II ♀	III ♂	III ♀
Body weight in grams less organs, less gastro-intestinal tract <sup>1</sup>	214.5	148.6	133.1	103.6	120.5	110.6
Mean hair size in millimeters at 460 days	0.073	0.066	0.060	0.053	0.054	0.051
Mean hair size in millimeters at 746 days	0.071	0.063	0.064	0.059	0.070	0.062
Mean volume of femur in cubic centimeters	0.605	0.445	0.422	0.353	0.445	0.379
Mean weight of femur in grams	0.741	0.540	0.486	0.398	0.484	0.432
Density of femur	1.22	1.21	1.15	1.13	1.09	1.14
Mean length of femur in centimeters	3.85	3.43	3.43	3.26	3.54	3.36
Mean smallest diameter of femur in centimeters	0.35	0.32	0.33	0.31	0.34	0.32
Mean weight of liver in grams <sup>2</sup>	11.49 (11)	10.06 (18)	9.33 (7)	6.82 (13)	8.55 (9)	8.72 (10)
Mean weight of kidneys in grams	3.29	2.58	2.06	1.78	2.17	1.96
Mean weight of spleen in grams	0.82	0.81	0.47	0.47	0.75	0.75
Mean weight of heart in grams	2.04	1.81	1.57	1.16	1.55	1.21
Mean body length (nose to anus) in centimeters	21.9	19.8	18.4	18.0	18.1	17.5

<sup>1</sup> The organs include those listed as well as the lungs and genital organs.

<sup>2</sup> The numbers in parentheses give the number of animals used for organ weights.

weight data are based is shown by the figures in parentheses after the mean liver weight data. These numbers also refer to the oldest animals in each of the groups of table 2.

With the exception of group III, the livers of the males are larger than those of the females. In group III they are

about the same. The livers are smaller in the retarded groups than in those that matured rapidly, no. I. In spite of the fact that these are the livers of very old animals, they bear a close relation to the body weight at the time of death. These body weights, less the gastro-intestinal tracts and less the organs given in this table plus the lungs, are shown in the first line of table 3. Many of these animals were thin and emaciated at the time of death.

The kidneys of the males are consistently larger than those of the other sex. The kidneys do not correspond to the body weights at the time of death according to the tables of Donaldson ('24). On the other hand, these kidneys correspond very closely to the maximum weights attained by these various animals. If the maximum body weights of these rats of groups I♂ to III♀, respectively, are calculated from the kidney weights by the use of Donaldson's tables, one gets values of 420, 325, 240, 215, 268 and 235 gm., while from table 1 one can secure the mean maximum weight values for the same groups. These prove to be 439, 355, 262, 210, 267 and 227 gm., respectively. The similarity of these values is striking. Two explanations are apparent. One is that the kidneys of all groups were enlarged at the time of maximum weight and have decreased proportionately as the animal body wasted away before death. In the light of the findings of McLennan and Jackson ('33), however, it is more likely that the kidneys remained very close to their maximum weight even when the animals died in extreme old age. The shrinkage of the liver also corresponds to the findings of these authors. Due to the high protein content of our diets some enlargement of these kidneys might have been expected.

The spleens of the opposite sexes in the three groups are the same in weight. The spleens of group II are small, but no explanation for this size is available.

The hearts of all animals are much larger than even the maximum weights of the animals would indicate. These values exceed the highest given by Donaldson. This indicates that these hearts were considerably enlarged at the time of

death. This may be part of the pathology of old age or it may be due to some constituent of our diets. In our laboratory it has been observed that the hearts of guinea pigs can be enlarged in the course of a few months by feeding cod liver oil at levels lower than that employed in these diets. It cannot be stated at this time if this is the responsible agent in this case.

The hearts of the females are consistently lighter than those of the males. The hearts of the retarded groups are also lighter than those of group I. These organ data strengthen the thesis that the retarded animals failed to attain the body size of those that matured rapidly. Inasmuch as the male organs in the retarded groups are larger than the corresponding ones of the opposite sex, in most cases, this affords some indication that these organs may have shared in the retention of the superior growth potential that characterized the males of the retarded animals.

### *Bone growth*

In order to study the growth of the bones in the three groups, all animals were preserved in formaldehyde. After the last animal had died, the femurs were removed. After these had dried in air the last traces of tissue were removed. The maximum length and the minimum diameter of each femur was measured.

Some of the femurs of the retarded groups proved to be very fragile. Some crumbled in the course of dissection. Part of these femurs from the retarded groups proved to be only thin cylinders of bone.

After the length and diameter were measured, each femur was weighed in air and then weighed suspended in water. The volume of each was then calculated. These measurements upon the femurs are included in table 4.

The bone measurements fit the general picture of the final size attained by the various groups. The bones of the males are consistently larger than those of the females in all groups. The bones of the retarded animals of the same sex are con-

siderably smaller than those of group I which grew to maturity rapidly. Inasmuch as the femurs of the males of the retarded groups are larger than those of the females there is some indication that the bones as well as the organs and the entire body shared in the final growth at the end of the long retardation. All measurements indicate that the femurs of group III were slightly larger than those of the same sex of group II. This suggests that some advantage in bone growth resulted from the initial gain in body weight which was allowed group III at the very beginning of the experiment.

As soon as the bones of groups II and III were weighed in water it was evident that they were less dense than those of group I. Some of the femurs of II and III floated. Some contained a small hole and immediately filled with water. These latter were discarded. In considering these data it will be observed that the bones from groups II and III were from extremely old animals while those from no. I were much younger.

The values for the density are the same for the opposite sexes, but the animals that grew to maturity early had bones of greater density. The capacity for bone growth may be lost earlier than that for other parts of the body.

#### *Maintenance requirements of retarded-growth animals*

The calories needed for maintaining the individuals of groups II and III at fixed levels of body weights were ascertained with accuracy for each. This was essential to maintain the body weights at fixed levels. The requirements at these various weight levels are summarized in table 4.

The females consistently require more calories for maintenance at the various weight levels.

The 't' values for these data were computed by the method of Livermore ('34). In group II the odds are 255:1 and in group III 9999:1 that the differences in calory requirements by the opposite sexes are significant.

Morris, Palmer and Kennedy ('33) found the female rat *M. musculus* more efficient than the male in the utilization of food.

Part of this inefficiency of the female sex can be attributed to the slower growth rate. Part may be due to this higher maintenance requirement which exists when both sexes are forced to maintain the same body weight.

TABLE 4

*Calory requirements for maintenance of the body weight at various levels*

GROUP II			GROUP III		
Body weight level	Average calories per day per 100 gm. rat		Body weight level	Average calories per day per 100 gm. rat	
	♂	♀		♂	♀
<i>gm.</i>			<i>gm.</i>		
40	33.4±0.69	34.7±0.19	80	26.8±0.80	27.7±0.75
50	28.6±1.16	30.4±0.97	90	22.6±0.63	23.5±0.67
60	25.0±0.72	27.3±0.52	100	21.1±0.58	22.2±0.58
70	21.0±0.34	22.5±0.27	110	18.3±0.26	19.6±0.28
80	21.9±0.33	23.5±0.25	120	18.7±0.26	20.2±0.23
90	20.5±0.52	21.4±0.48	130	19.4±0.30	20.3±0.43
100	20.6±1.06	21.9±0.55	140	18.4±0.23	19.2±0.24
110	18.4±1.00	18.8±0.26	150	16.9±0.35	16.6±0.24
120	20.9±0.18	20.8±0.52	160	19.0±0.33	20.7±0.54
130	20.9±0.46	20.1±0.45	170	18.3±0.57	19.5±0.64

TABLE 5

*Gross pathology at the time of death, in per cent of entire group<sup>1</sup>*

GROUP NO.	LUNGS	KIDNEYS	GENITAL TRACT	TUMORS
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
I ♂	78.0	14.0	...	14.0
I ♀	54.0	36.0	45.0	18.0
II ♂	58.0	16.0	8.0	8.0
II ♀	35.0	9.0	22.0	22.0
III ♂	85.0	50.0	...	7.0
III ♀	35.0	8.0	15.0	10.0

<sup>1</sup> This table is based on the number of animals in any one group showing definite types of infections on post mortem examination.

### *Pathology at the time of death*

In the course of nearly 4 years in which this experiment was in progress many different pathological conditions were observed that are rarely seen in rat colonies. This was due to the maintenance of old animals in contrast to the usual

breeding colony for rats where individuals are usually discarded shortly after middle life.

The roughness of the fur coats of the old animals became apparent much earlier in those that matured rapidly. This is well illustrated in the photograph included in our preliminary report. As the experiment progressed many animals became blind. This happened so gradually that no quantitative data were secured, but a rough estimate would include at least half of the animals that lived beyond 2 years.

Old rats were frequently afflicted with diseases of the urinary tract. At times bloody urines were observed. In some cases individuals were treated with hexamethylenetetramine, but such treatment was usually futile. The old rat undoubtedly is a fertile experimental animal for the urologist.

In table 5 an attempt has been made to summarize some of the observations of the gross pathology at the time of death. The failure of the lungs is obvious in many cases, but this must have been secondary to many of the other failures that occurred in the old bodies.

#### SUMMARY

Rats were retarded in growth and not allowed to attain maturity until after periods of 766 and 911 days. The rat body still retains the power to grow at these extreme ages. After such periods of retardation the rat cannot attain a body size equal to that of an animal that grows to maturity younger. This conclusion is based upon the smaller size of the entire body, the weight of such organs as the heart, and the size of the bones represented by the femur. Even after these long periods of suppressed growth the male rat retains a growth potential greater than the female although the males of the retarded groups grow no larger than the normal females of this species. The hearts of all these animals dying in old age were larger than normal while the livers were smaller. The kidneys corresponded in weight at the time of death to the maximum weight attained by the body. The femurs of members of the retarded groups were less dense than those that matured normally.

In both retarded groups individuals of both sexes attained extreme ages beyond those of either sex that grew normally. The mean age of the males of both retarded groups was greatly increased in comparison with 'rapid growth' males while the mean age for the females was about the same in all three groups. The males of the retarded groups exceeded the females in age in contrast to the 'rapid-growth' group.

At a constant weight level in the course of retarded growth the female requires more calories for maintenance than the male. In the course of retarded growth, the diameter of the hair as well as the growth of the body reflects the retardation.

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# VARIATIONS IN URINARY REDUCING SUBSTANCES OF TWO NORMAL DOGS MAINTAINED ON BREAD DIETS <sup>1</sup>

EDWIN P. LAUG AND THOMAS P. NASH, JR.

with the technical assistance of Louise A. Garavelli

*Department of Chemistry, School of Biological Sciences, University of Tennessee,  
Memphis*

## TWO FIGURES

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Procedures which are employed to determine the gross availability of carbohydrates for animal nutrition fail to distinguish the more subtle changes which may occur in the carbohydrates of foods prepared by cooking, baking, canning, and other treatments. Regardless of conflicting interpretations<sup>2</sup> of the metabolic significance of sugar-like reducing substances in normal urine, further knowledge of the relations between diet and these normal reducing substances of urine should aid in the evaluation of the carbohydrates.

<sup>1</sup> This study was supported by a grant from Standard Brands, Inc.

<sup>2</sup> Benedict and Osterberg ('18), Benedict, Osterberg and Neuwirth ('18), Benedict and Osterberg ('23) and Neuwirth ('22) believe that there is a 'glycuresis' or excretion of sugar in normal urine intimately related to the ingestion of food. Folin and Berglund ('22) claim that the glycuresis represents not utilizable sugar but carbohydrate moieties from food rendered unassimilable by processes of cooking and baking. In this latter opinion, Greenwald, Gross and Samet ('25) concur. Recently West, Lange and Peterson ('32) have supported the original 'glycuresis' idea of Benedict with results that indicate a very small quantity of fermentable material (glucose?) is always present in urine even during fasting. This fasting excretion of fermentable material is denied by Harding and Selby ('33) and the present authors (Laug and Nash, '35) have cited some experiments which make it seem likely that a portion of the so-called fermentable material is formed from a precursor by hydrolysis during preparation of the urine filtrates with acid  $\text{HgSO}_4\text{-BaCO}_3$ .

We therefore report experiments in which we have examined total, fermentable, non-fermentable, and hydrolysable reducing substances in normal urines upon diets restricted to bread.

#### METHODS AND PROCEDURES

Breads were obtained from two local bakeries having a large distribution. (In the tables the source of the bread is indicated as 'K' and 'W'.) Because of variations in moisture content which would render comparisons from day to day difficult, the sliced breads were dried to constant weight, without toasting, in a specially constructed warm air drier (Laug, Garavelli and Nash, '34). An average loss of 35 per cent of the fresh weight occurred. The dried slices were then finely ground either whole, or, for some experiments, after the crust and white inside portions had been separated.

Analyses of the dried bread powders are given in table 1. Total carbohydrate was determined by Allihn's method after initial hydrolysis of starch by Taka-Diastase and subsequent mild hydrolysis by HCl (Olmsted, '20). The carbohydrate material amenable to Taka-Diastase hydrolysis was somewhat less in whole wheat and rye breads than in wheat bread. Free sugar in one sample of wheat bread amounted to 9 per cent.

Two hundred and fifty grams of the bread powder were moistened with water to make a thick mush and fed once daily to each of two female dogs of approximately equal weight (17 kilos). Dog I, a young police dog about a year old, gained weight slowly over the diet period of 3½ months. Dog II, a mongrel of bull dog type, maintained a steady weight throughout the diet period of 4 weeks. Except for the first few days, when the bread diet was instituted, the animals exhibited normal appetite and ate with great relish. No constipation developed, except over a 3-day regime during which one dog received only the white inside portion of the bread. A mildly laxative action of the crust was clearly exhibited. Both animals were alert and showed all signs of well-being.

Urine collections were made by catheter, with adequate washing of the bladder at intervals of 2 to 4 hours throughout a 12-hour period after feeding. Moderate pains were taken to maintain sterile technic, at no time was there ever any evidence of bladder infection. All urines were acid to litmus save those collected in the first post feeding period, when a slight alkaline tide usually occurred. Urines were preserved with toluene and kept on ice.

Urine filtrates were prepared by the acid  $\text{HgSO}_4\text{-BaCO}_3$  procedure of West and Peterson ('32). Reducing materials

TABLE 1  
*Analyses of the dried bread used in the diet experiments*

KIND OF BREAD	MOISTURE CONTENT	PROTEIN <sup>1</sup>	TOTAL CARBOHYDRATE AS GLUCOSE
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
K. Wheat	0.8	15.75	79.0
K. Wheat (crust)	1.6	15.13	81.8 <sup>2</sup>
K. Wheat (white inside)	1.8	15.25	82.8 <sup>2</sup>
W. Wheat	0.5	14.25	78.1
W. Rye	1.2	13.50	76.8
W. Whole wheat	1.3	15.20	72.7

<sup>1</sup> Protein = total nitrogen  $\times$  conversion factor 6.25.

<sup>2</sup> By direct mineral hydrolysis of starch, instead of by Taka-Diastase.

in the filtrates were determined with the sensitive Shaffer-Hartmann reagent no. 50, containing 1 gm. of KI per liter (Shaffer and Somogyi, '33). Fermentation technic was that of Somogyi ('27). Hydrolysis of filtrates was accomplished by making them approximately 1 N with  $\text{H}_2\text{SO}_4$  and heating on the water bath for  $2\frac{1}{2}$  hours. After cooling the hydrolysate and diluting with water to 50 ml. volume, the mixture was neutralized with dry  $\text{BaCO}_3$ .

#### EXPERIMENTAL

It has been known for some time that ingestion of carbohydrates degenerated by heat produces a large increase in urinary reducing substances. Particularly is this true of

such food products as caramelized sugars, Karo syrup, Grape Nuts, and even ordinary toast. Folin and Berglund ('22) have postulated that a considerable part of the reducing materials excreted in urine does not reflect the course of events in carbohydrate metabolism but represents merely the escape of carbohydrates rendered unassimilable by processes of cooking and baking. Figure 1 compares the results of feeding the white inside and crust parts of bread in daily 250 gm. portions. A considerable rise in the total reducing values after crust feeding reflects chiefly the increase in the non-fermentable fraction. The slight rise in the fermentable values may be due to hydrolysis during preparation of the urine filtrates by the strongly acid mercuric sulphate reagent (Laug and Nash, '35). *Pari passu* with the increase in non-fermentable reducing substances is the well-marked rise in total nitrogen. It is particularly interesting to note that the output of reducing and nitrogenous substances is exceedingly slow. With either type of food, the 4-hour periods 8 hours after feeding still show a maximal excretion even of the fermentable fraction alleged to be glucose. Höst ('23) regards such time relations as evidence that excretion of reducing material does not represent normal carbohydrate metabolism; he has shown that the rise of such materials in the blood is prompt and precedes by several hours the rise in the urine. Likewise, Folin and Berglund ('22) say: "Since the sugar of normal urine is quite independent of the level of the blood sugar, it must be considered exceedingly doubtful whether the sugar of normal urine is glucose, quite independently of whether it does or does not ferment." Wang and Felsner ('24) found in the urine considerable increases in reducing materials after feeding discolored commercial glucose, whereas alcoholic extracts of the brown coloring material containing presumably denatured carbohydrates, produced no rise in blood sugar. It may be that the kidneys effect a relatively high concentration of non-fermentable (non-assimilable?) substances from the blood (West, Lange and Peterson, '32). It is, of course, also possible that the digestive function of the dog may be very slow or deficient in respect to

starch. But we have observed (Laug, Garavelli and Nash, '34) that in the phlorhizinized dog a delayed excretion of sugar is not evident. In this type of renal diabetic there is a sharp rise in excretion 2 to 3 hours after feeding bread, followed by a rapid return to a low level. The question may be raised whether in the phlorhizinized animal there is an enhanced process of assimilation of sugar from starches to meet the urgent need of the body, or whether in the normal animal

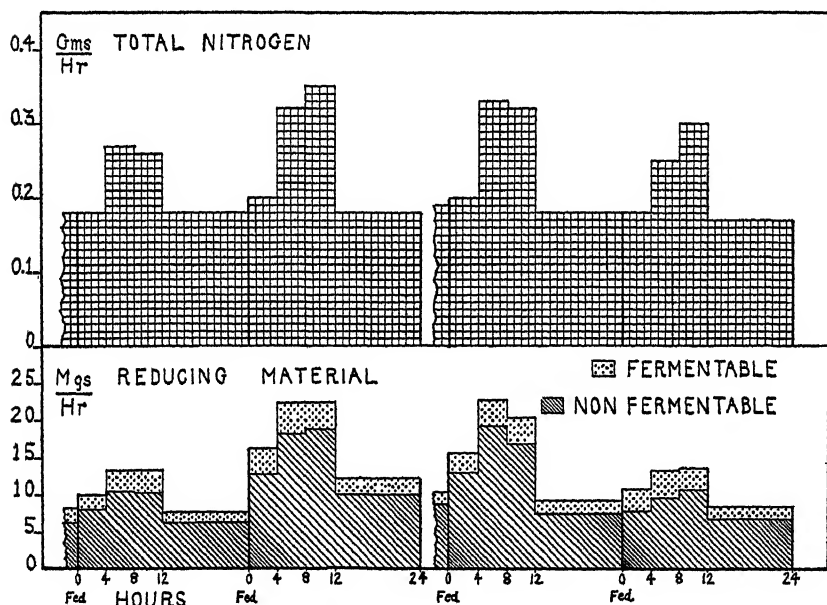


Fig.1 Excretion of reducing material and total nitrogen in the urine of dog I after feeding white inside (first and last periods) and crust (second and third periods) of wheat bread.

the slow excretion is a reflection of unknown factors in carbohydrate metabolism. We are inclined to the latter view.

In summarizing seven 24-hour periods (four of which appear in fig. 1), it was found that on 4 days of white inside feeding, the averages of reducing materials excreted were: Total, 235.4 mg. (range, 211-246); non-fermentable, 182.9 mg. (range, 166-192) and fermentable, 52.3 mg. (range 45-59). During 3 days of crust feeding the averages were: Total,

364.2 mg. (range, 346–386); non-fermentable, 301.5 mg. (range, 286–318); and fermentable, 62.7 mg. (range, 60–68). Of the average increase in total, (364.2 minus 235.4 equals 128.8 mg.), 92 per cent was accounted for by the increase in the non-fermentable and only 8 per cent by the fermentable fraction.

Figure 2 describes the excretion of reducing substances for 5 successive days during which wheat bread, rye bread, and

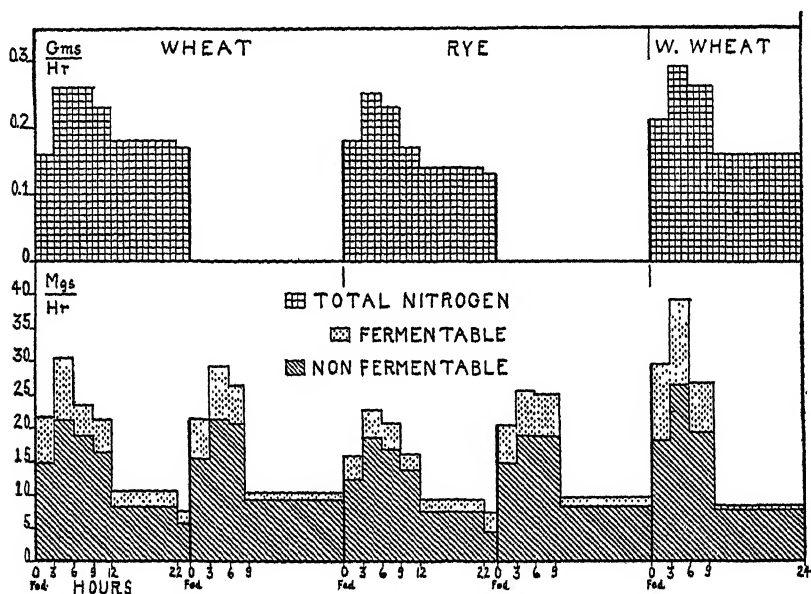


Fig. 2 Excretion of reducing material and total nitrogen in the urine of dog II after feeding wheat, rye, and whole wheat bread.

whole wheat bread were fed in rotation. The results indicate that the excretion of non-fermentable and fermentable materials is lowest after feeding rye bread, intermediate after wheat bread, and highest after whole wheat bread. While fermentable materials are always present, even in 2-hour urines preceding feeding, the value falls almost to zero during the night after the whole wheat bread meal. Gross analyses of breads (vide table 1) are unable to define such distinctions, except to indicate that whole wheat and rye breads contain somewhat less starch and more fibrous substance.

TABLE 2

The excretion of reducing materials (before and after hydrolysis) in the urine of dog I after feeding three different bread preparations

DATE 1934	URINE COLLECTION	URINE VOLUME	REACTION TO LITMUS	TOTAL N	REDUCING SUBSTANCES			REDUCING SUBSTANCES PRODUCED BY HYDROLYSIS		NON-FERMENTABLE REDUCING SUBSTANCES						
					Total	Fermentable		Total	Fermentable	Before hydrolysis	After hydrolysis					
4/5  fed 250 gm. wheat bread (K) at 10.00 A.M.	10.00 A.M.-12.00 M. 12.00 M. - 3.06 P.M. 3.06 P.M.- 7.30 P.M. 7.30 P.M.- 8.00 A.M. 8.00 A.M.-10.00 A.M.	cc.	S. alk. S. alk. Acid Acid	Gm. hour 0.13 0.21 0.41 0.19 0.10	Mg. hour	Per cent of total	Mg. hour	Mg. hour	Mg. hour	Mg. hour						
					7.9	22.3	3.8	2.9	6.1	7.1						
					11.5	13.5	7.0	6.7	9.9	10.2						
					13.0	19.7	5.8	6.4	10.5	9.8						
					8.2	19.9	3.9	4.0	6.6	6.7						
					4.0	8.4	2.0	1.7	3.5	3.9						
Totals for 24 hours					219.1	39.9	18.2	106.9	108.1 (101% of total)	178.8	178.3					
4/6  fed 250 gm. wheat bread (K) at 10.00 A.M.	10.00 A.M.- 1.00 P.M. 1.00 P.M.- 5.00 P.M. 5.00 P.M.- 9.06 P.M. 9.06 P.M.-10.00 A.M.	25 60 70 310	S. alk. S. acid Acid Acid	0.17 0.24 0.26 0.22	7.5	22.3	3.6	2.8	5.9	7.7						
					11.0	19.1	8.3	7.2	8.9	10.1						
					12.8	21.0	8.8	7.6	10.1	11.3						
					8.1	14.9	3.3	3.2	6.9	7.1						
					Totals for 24 hours					223.1	40.0	17.9	123.6	109.0 (88.2% of total)	183.2	197.7
					4/7  fed 250 gm. crust from wheat bread (K) at 10.00 A.M.	10.00 A.M.-12.00 M. 12.00 M. - 3.00 P.M. 3.00 P.M.- 7.30 P.M. 7.30 P.M.-10.10 A.M.	8 30 95 312	Acid Acid Acid Acid	0.14 0.22 0.30 0.19	8.6	15.5	5.8	5.3	7.2	6.6	
15.8	18.1	7.2	7.6	13.0						12.5						
20.2	20.7	7.7	8.6	16.0						15.1						
11.0	17.7	5.0	4.6	9.1						9.6						
Totals for 24 hours										316.9	57.8	18.2	141.3	138.9 (98.3% of total)	259.2	261.6
4/16  fed 370 gm. fresh wheat (K) bread & 250 gm. dried at 9.00 A.M.	9.00 A.M.-11.00 A.M. 11.00 A.M.- 2.00 P.M. 2.00 P.M.- 5.00 P.M. 5.00 P.M.- 8.00 P.M. 8.00 P.M.- 8.00 A.M.	30 45 114 87 435	S. alk. Alk. Acid Acid Acid	0.18 0.26 0.31 0.29 0.18						9.0	24.7	4.6	4.3	6.8	8.4	
					11.4	25.2	6.6	5.7	8.5	9.5						
					11.5	26.9	8.2	6.7	8.4	9.8						
					11.3	25.3	7.4	6.0	8.5	9.8						
					5.9	21.0	2.8	2.2	4.7	5.5						
					Totals for 23½ hours					198.8	47.5	24.0	111.0	87.8 (79.2% of total)	151.3	173.5

<sup>†</sup> In cases where the fermentable appears higher than the total fraction after hydrolysis, it indicates that some of the non-fermentable material has contributed, by being itself slightly hydrolysed.



Table 2 presents in addition to the total and fermentable reducing substances in urine, data on the reducing substances produced by hydrolysis, after diets of whole bread, fresh and dried, and crust. Before discussing these it should be noted that the presence of crust (about 19 per cent) in whole bread appears to account for an increase in the non-fermentable fraction over the period when the diet was crust free (white inside). Compare 219.1 and 223.1 mg. after whole bread, with the average 182.9 mg. on white inside. Also, while toasting has never occurred in our process of drying bread, it is interesting to note that when fresh bread in amount equivalent to dry bread is fed, the distribution of fermentable and non-fermentable reducing substances in the urine indicated an increase in the former fraction and a decrease in the latter.

The total hydrolysable fraction follows rather closely total reducing values. After feeding, the two rise and reach their highest levels at approximately the same time. The hydrolysable value never falls to zero, even 22 hours after feeding. The fermentable and non-fermentable products of hydrolysis are distributed, however, quite differently from the non-hydrolysed. In agreement with observations of West, Lange and Peterson ('32) and Patterson ('26) practically 100 per cent of the hydrolysed material is fermentable. The hydrolysed substance is not produced at the expense of the original non-fermentable reducing fraction. The data of table 2 show no significant change in value of the non-fermentable fraction after hydrolysis.

Edwards and Everett ('34) in their experiments on toast and Grape Nut feeding to man find no increase in the hydrolysable fraction but in most cases observe destruction of reducing material by hydrolysis. If the substances of bread crust are the same as those produced by toasting our results are not in accord with those of Edwards and Everett. Indeed, we find after crust feeding a slightly larger hydrolysable fraction than after whole bread feeding. Greenwald, Gross, and McGuire ('27), upon feeding heated sugar found no increase in non-fermentable reducing substances in the urine of man,

TABLE 3  
The excretion of reducing materials (before and after hydrolysis) in the urines of dogs I and II after feeding wheat and whole wheat bread

DATE 1934	URINE COLLECTION	URINE VOLUME cc.	REACTION TO LITMUS	TOTAL N	REDUCING SUBSTANCES PRODUCED BY HYDROLYSIS			NON-FERMENTABLE REDUCING SUBSTANCES	
					Total	Fermentable	Mg. hour	Before hydrolysis	After hydrolysis
Dog II 5/28 fed 250 gm. wheat bread (W) at 10.10 A.M.	10.00 A.M.- 1.00 P.M.	47	Neut.	0.24	18.3	4.2	25.2	4.3	12.9
	1.00 P.M.- 4.00 P.M.	125	Sl. acid	0.31	28.7	8.6	30.1	20.1	19.0
	4.00 P.M.- 7.00 P.M.	138	Acid	0.30	24.3	6.8	28.5	17.4	14.3
	7.00 P.M.-10.06 P.M.	100	Acid	0.23	16.4	3.2	20.0	13.1	11.5
	10.06 P.M.- 8.00 A.M.	267	Acid	0.14	6.7	0.67	10.1	6.1	6.6
Totals for 24 hours					341.1	77.1	22.6	264.0	250.5
Dog II 5/29 fed 250 gm. whole wheat bread (W) at 10.10 A.M.	10.00 A.M.- 1.00 P.M.	40	Neut.	0.21	27.9	11.2	40.0	11.8	15.7
	1.00 P.M.- 4.00 P.M.	110	Acid	0.36	41.2	13.3	32.5	27.7	26.0
	4.00 P.M.- 7.03 P.M.	117	Acid	0.32	26.7	6.5	24.2	20.3	21.0
	7.03 P.M.-10.00 P.M.	75	Acid	0.22	13.8	4.4	32.3	9.5	13.1
	10.00 P.M.- 8.00 A.M.	192	Acid	0.16	6.6	0.12	1.8	6.4	7.9
Totals for 24 hours					405.0	107.3	26.5	301.8	326.8
Dog I 5/28 fed 250 gm. wheat bread (W) at 10.20 A.M.	10.15 A.M.- 1.15 P.M.	35	Neut.	0.22	14.7	5.1	35.0	9.5	8.2
	1.15 P.M.- 4.15 P.M.	35	Acid	0.27	23.7	7.4	31.0	16.3	14.1
	4.15 P.M.- 7.15 P.M.	57	Acid	0.26	24.3	7.2	29.5	17.1	17.8
	7.15 P.M.-10.21 P.M.	52	Acid	0.24	19.2	5.3	27.4	14.0	15.2
	10.21 P.M.- 8.15 A.M.	328	Acid	0.18	10.7	2.3	21.6	8.5	10.2
Totals for 24 hours					365.3	101.4	27.8	263.9	282.7
Dog I 5/29 fed 250 gm. whole wheat bread (W) at 10.20 A.M.	10.15 A.M.- 1.15 P.M.	32	Acid	0.17	24.4	11.0	44.8	13.5	12.9
	1.15 P.M.- 4.15 P.M.	87	Acid	0.29	37.9	12.0	31.7	25.9	23.3
	4.15 P.M.- 7.15 P.M.	82	Acid	0.30	31.4	13.7	43.4	17.8	21.0
	7.15 P.M.-10.15 P.M.	55	Acid	0.25	21.9	5.1	23.1	16.9	17.8
	10.15 P.M.- 8.15 A.M.	133	Acid	0.16	10.4	1.6	15.1	8.9	11.4
Totals for 24 hours					464.1	141.7	30.5	321.0	326.2

but a considerable increase in the dog with a rise in the hydrolysable fraction. It is possible that species differences in excretion and nature of urinary reducing substances may be more important than have been recognized.

The major interest in the data of table 3 is the contrast in results obtained upon diets of wheat bread and whole wheat bread, respectively. The total reducing urinary output is considerably higher upon the whole wheat bread feeding. Of the total increase, 47 per cent (dog II) and 41 per cent (dog I) occurs in the fermentable fraction. This behavior is quite different from that obtained after crust feeding, where the chief rise is in the non-fermentable fraction. If the increase in the non-fermentable fraction after crust feeding is any indication of the presence in the blood of non-utilizable sugar-like substances, the urine picture after whole wheat bread feeding might be expected to be qualitatively similar. In both dogs the total hydrolysable material is decreased about 50 per cent upon the whole wheat bread diet. During several periods the hydrolysable fraction falls practically to zero, and in one case there is actual loss of reducing value associated with hydrolysis. The fermentable fraction of the total hydrolysable material is considerably smaller. This is also apparent from the increase in the non-fermentable values after hydrolysis. In terms of an hypothesis proposed elsewhere (Laug and Nash, '35), these findings indicate that hydrolysis was not completed in all cases, with the resulting accumulation of the first stage of hydrolysis material which is not fermentable. An unexplained effect is noted in the behavior of the fermentable fraction of the night and following morning samples of urine after whole wheat bread feeding; this becomes vanishingly small or disappears entirely.

Although the analyses of breads from the two different bakeries show essentially agreeing values for total carbohydrates, the feed-experiments on dog I which received these two kinds of bread (compare tables 2 and 3) indicate differences in excretion of reducing materials. The level of excretion of total, non-fermentable, and fermentable after bread W

was higher than with bread K. But the distribution of fermentable and non-fermentable substances was different. With bread W the fermentable fraction was higher (27 per cent as compared with 18 per cent). This difference is probably accounted for in the methods of baking. No significant differences in the total hydrolysable fractions are apparent.

After feeding, the hourly rate of excretion in dog II reached a peak higher and usually more rapidly than in dog I; while dog I, excreting more slowly, put out a larger amount in the whole period.

#### SUMMARY

A study has been made of the excretion of reducing substances in the urine of two normal dogs maintained on bread diets.

Significant variations in the total, fermentable, non-fermentable, and hydrolysable reducing substances can be correlated with the particular kind of bread diet.

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# THE INFLUENCE OF CHRONIC FLUORINE TOXICOSIS IN LAYING HENS UPON THE FLUORINE CONTENT OF THE EGG AND ITS RELATION TO THE LIPOID CONTENT OF THE EGG YOLK<sup>1</sup>

PAUL H. PHILLIPS, J. G. HALPIN AND E. B. HART

*Departments of Agricultural Chemistry and Poultry Husbandry,  
University of Wisconsin, Madison*

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In a previous study (Phillips, Hart and Bohstedt, '34) it has been shown that fluorine in the ration of dairy cows does not influence the nutritional qualities of milk. In so far as we could determine chemically and biologically, there was no significant increase in fluorine content of the milks produced. The chicken has been shown to be more resistant to fluorine toxicosis than other species (Halpin and Lamb, '32). This knowledge is very apt to lead to over confidence in the use of fluorine containing mineral supplements—since it develops a feeling of safety with respect to poultry products for human consumption. It was with this problem in mind that a study of the fluorine content of the eggs from chronically poisoned laying hens was undertaken.

Most of the studies reported in the literature concerning the effects of fluorine in the nutrition of poultry deal with the levels of tolerance and the physiological responses to fluorine ingestion. Puresz et al. ('34) reported the appearance of fluorine in the egg following intravenous injections of 30 micrograms of NaF every fifth day. The fluorine content was reported to have reached the maximum value of

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46.3 micrograms per cent. This gradually receded upon the discontinuance of the injections. No fluorine was found in the normal egg although the method was reported to be sensitive to fluorine in quantities of 25 to 400 micrograms. Continued injection of F retarded egg production and there was a gradual loss of egg weight. The data reported covered only two birds and the period of time was relatively short since it covered only  $2\frac{1}{2}$  months.

#### EXPERIMENTAL

The animals used in these studies were birds grown on rations designed to study the influence of fluorine. They had almost finished their second laying year at the time the eggs were collected for study. They had been on their experimental rations and subjected to the chronic influence of fluorine for approximately 28 months.

The rations used were similar to those previously used and described elsewhere (Halpin and Lamb, '32). The basal ration was composed of yellow corn 65 parts, wheat middlings 16 parts, dried skim milk 18 parts, and salt 1 part. Lot I received this ration unsupplemented by mineral additions. Lot II received 95 parts of the basal ration with 2 parts of ground limestone and 3 parts of steamed bone-meal as a mineral supplement. The rations of lots III, IV, and V were identical with that of lot II except 1 per cent, 2 per cent, and 3 per cent of raw rock phosphate was substituted, respectively, for like quantities of steamed bone meal. Except for lot I, the rations contained approximately 1.98 per cent Ca and 0.91 per cent P, thus providing a calcium:phosphorus ratio of about 2:1.

The eggs were collected during the months of July and August. They were stored until fluorine determinations could be made. Three to 4 dozen eggs were obtained for each lot. A batch of twelve eggs was used for each set of determinations made. In preparation for analysis twelve eggs from each respective lot were washed in distilled water, then hard boiled and separated into three portions, shell, yolk, and

white. These were dried and quadruplicate samples were taken for analyses. Duplicate samples were run without added fluorine and to each of the other two samples 0.01 mg. of F was added. The samples were ashed repeatedly with  $\text{Na}_2\text{CO}_3$  at low temperature until a white ash was obtained. The ash was then analyzed for F by the method of Willard and Winter ('33) using sodium alizarin sulphonate as the indicator.

#### RESULTS

The first analyses showed that little or no fluorine was present in the egg shell proper, but the whole egg from the lots receiving rock phosphate contained increased quantities of fluorine. A separation of the egg into the yolk and white indicated that traces of F only were present in the white. With this information at hand effort was concentrated upon the egg yolk. An attempt was made to determine with what constituents of the egg yolk the F was carried or combined.

The yolks of twelve eggs were continuously extracted by ether for 24 hours to obtain the ether soluble substances in an attempt to divide the yolk into three fractions, true fat, lipid substances, and non-fat materials. It was found that ether extraction did not completely remove the fatty constituents. However, if ethyl alcohol was substituted for the ether and the extraction continued for 48 hours practically complete removal of the fatty material was accomplished. The alcohol from the alcohol extract was then removed by drying on a sand bath. The alcohol residue was next extracted repeatedly with anhydrous acetone until all acetone soluble products were removed. The acetone insoluble residue was next extracted with ether. Thus the yolk was roughly partitioned into three portions: a non-fat or alcohol insoluble residue, 31 to 38 per cent; a fat or an acetone soluble fraction, 42 to 48 per cent, and the acetone residue soluble in ether or the complex lipids, 15 to 25 per cent. Each fraction was then analyzed for its fluorine content. The results are summarized in table 1.



Examination of table 1 indicates clearly that the inclusion of 0.035 to 0.105 per cent F in the ration in the form of rock phosphate causes an increase in the quantity of fluorine occurring in the egg yolk. Further, the fluorine seems almost completely to accompany the lipid fraction of the yolk.

TABLE 1

*The fluorine content of various constituents of the egg as affected by chronic F toxicosis in laying hens (milligrams of F per 100 gm.)*

		WHITE	YOLK	YOLK- ACETONE RESIDUE (LIPOID FRACTION)	YOLK- ACETONE SOLUBLE FRACTION (FAT)	YOLK- ALCOHOL INSOLUBLE RESIDUE (NON-FAT)
Lot I no mineral	Average of samples without added F	0.02	0.09	....	None	None
	Average of samples calculated after deducting added F	0.03	0.08	....	None	None
Lot II lime-stone and bone meal	Average of samples without added F	0.02	0.12	0.17	None	None
	Average of samples calculated after deducting added F	....	0.12	0.16	None	None
Lot III 1 per cent rock phosphate (0.035 per cent F)	Average of samples without added F	0.02	0.18	0.27	Trace	None
	Average of samples calculated after deducting added F	0.01	0.20	0.29	Trace	None
Lot IV 2 per cent rock phosphate (0.07 per cent F)	Average of samples without added F	0.02	0.33	0.69	Trace	None
	Average of samples calculated after deducting added F	0.01	0.27	0.65	Trace	None
Lot V 3 per cent rock phosphate (0.105 per cent F)	Average of samples without added F	0.03	0.30	1.14	0.06	None
	Average of samples calculated after deducting added F	0.05	0.32	1.14	0.04	None

Traces of fluorine occurred in the fats and particularly at the 0.105 per cent level of fluorine feeding. This may be due to incomplete separation of the fats and lipoids. A relatively small amount of fluorine was found in the egg white. This is

believed to be the result of incomplete separation of the white and yolk after boiling and the values are therefore due in part at least to the lipoids of the yolk.

No change in egg size was noted in the course of these experiments. It seems that egg size is a genetic factor since severe fluorine toxicosis completely inhibited egg production rather than affected the size of the egg.

#### DISCUSSION

These results indicate that fluorine occurs in the whole egg yolk. Since the acetone insoluble fraction was approximately 20 per cent, it would be expected that the lipid portion would contain four to six times more fluorine than in the yolk itself. We are unable to account for this discrepancy. It is possible that the acetone extraction of the fatty materials was not complete for this would tend to reduce the lipid portion in the samples because of the true fat contamination, or that some of the fluorine was lost in the treatment of the egg yolks. The latter does not seem likely. However, for the purposes of this study, the results are sufficiently clear to demonstrate the accumulation of fluorine in the egg, primarily in the yolk, and that the fatty constituents of the egg yolk contains nearly all of the fluorine. Further, the evidence distinctly indicates that the fluorine is combined with the acetone insoluble fraction, or the lipoids of the egg yolk.

It is interesting to note that the evidence presented here indicates another halogen-lipoid combination in biological material, a fact which was recently emphasized by Peters and Man ('34).

It is desired to point out that the amounts of fluorine reported here may be of concern to the public health. On the basis of these results 3 to 5 dozen eggs from hens fed 3 per cent of rock phosphate would furnish about 1 mg. of fluorine. Recently Smith ('35) has shown that it requires only 1 mg. of F per liter of drinking water to cause the mottling of teeth. While the source of danger of fluorine toxicity from the egg is not comparable to sources in drinking water, the

universal use of eggs in the human diet, and especially for growing children, suggests the need of caution in the widespread use of fluorine containing mineral supplements in poultry feeding.

#### SUMMARY AND CONCLUSIONS

The addition of fluorine in the form of rock phosphate to the rations of laying hens results in a distinct and measurable increase in the fluorine content of the eggs. The fluorine accompanies the constituents of the egg yolk. It is completely separated from the non-fat portion of the yolk by extraction of the fatty constituents with ether or ethyl alcohol. It appears that the fluorine wholly or in large part at least, remains with the acetone-insoluble portion of the fat-like substances of the egg yolk. This suggests that fluorine is deposited in the egg in combination with the complex lipoids of the yolk.

It appears that fluorine is present in minute quantities in the normal egg. Increased fluorine ingestion did not reduce the size of the eggs in this series of experiments. The possibility of harmful effects arising from eggs from fluorine fed hens is discussed.

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## EDITORIAL REVIEW <sup>1</sup>

### THE CHIEF SULFUR COMPOUNDS IN NUTRITION

HOWARD B. LEWIS

*Department of Physiological Chemistry, Medical School, University of Michigan,  
Ann Arbor*

As an introduction to this review, a brief summary of the occurrence and chemistry of the sulfur compounds to be discussed and of the methods used in their study may be of value. Such a summary is presented in table 1 and will be commented upon briefly. Since the author has previously reviewed the earlier studies on the economy of sulfur ('24, '32, '33), the literature citations include for the most part only the more recent investigations.

Most important of the sulfur compounds of biological importance are the proteins which vary widely in their content of sulfur. On the one hand, we have the sulfur-poor proteins, as, for example, gelatin with a sulfur content of approximately 0.2 per cent, and many vegetable proteins, and on the other, the proteins of the epidermal tissues of the higher forms, the keratins, in some of which, as in human hair, the percentage of sulfur is more than 5 per cent.

In 1899, Mörner showed that this protein sulfur existed, in part at least, as the amino acid, cystine, discovered in 1810 by Wollaston as a constituent of a rare type of urinary calculus. It was soon recognized, however, that in a large number of proteins, even when the inadequacy of the existing analytical methods for the determinations of cystine was

<sup>1</sup>A revision of a paper presented before the Section on Medical Sciences of the American Association for the Advancement of Science as a part of a symposium on the "Chemistry and Metabolism of the Sulfur-containing Compounds of the body." Pittsburgh, December 28, 1934.

taken into consideration, the sulfur must also be present in some form other than cystine. In 1923, Mueller isolated a new type of sulfur-containing amino acid from protein hydrolysates, the structure of which was proven and the synthesis accomplished by Barger and Coyne in 1928. To the new sulfur-containing constituent of the protein molecule, the name methionine was given. The presence of methionine in

TABLE 1

*Sulfur compounds in the living organism*

- A. Naturally occurring organic sulfur compounds containing nitrogen.
  1. Proteins (0.2 to 5.0 per cent S).
  2. Known constituents of the protein molecule—The sulfur-containing amino acids.
    - a. Cystine—Wollaston—1810—calculi—Mörner—1899—protein.
    - b. Methionine—Mueller—1923—Barger and Coyne—1928.
  3. Probable derivatives of protein or of the above amino acids.
    - a. Taurine (taurocholic acid).
    - b. Cysteine.
    - c. Ergothioneine.
    - d. Glutathione (reduced and oxidized).
    - e. Insulin.
    - f. Mercapturic acids.
- B. Compounds related to or derivatives of naturally occurring sulfur compounds (the biological significance of these remains to be proven).
  1. Homologues of cystine or cysteine.
    - a. Homocystine (homocysteine).
    - b. Pentocystine (pentocysteine).
  2. Homologues of methionine.
    - a. Homomethionine.
  3. Thiolhistidine.
  4. Products of partial oxidation of cystine and cysteine.
    - a. Cysteic acid.
    - b. 'Suboxidation products' of cystine and cysteine (Toennies-Hammett).

practically all types of proteins has now been demonstrated although the quantitative data are inadequate (Baernstein, '32 b). In some proteins, however, notably in casein, methionine is present in amount far in excess of the amount of cystine. Despite much experimental study, the isolation of other sulfur-containing amino acids from protein has not been accomplished.

Numerous other sulfur compounds of great biological interest are known to occur in the animal world, many of which appear to be derivatives of protein or of the amino acids present in the protein molecule. Of these, taurine, isolated as a hydrolysis product of the bile acids by Gmelin, has been known for more than a century. Friedmann postulated the formation of taurine in vitro by oxidation of cystine to cysteic acid and decarboxylation of the latter but recent workers have found difficulty in effecting the second reaction of this synthesis, nor is the derivation of taurine from cystine in vivo definitely established.

Cysteine may be readily formed from cystine by reduction ( $RS-SR \rightleftharpoons 2 RSH$ ). In view of the interest in the function of the sulfhydryl group and of the discovery of glutathione, of which cysteine is a constituent, it is important to determine whether cysteine exists as such in the protein molecule or as its oxidation product, the disulfide, cysteine. Because of the ready oxidation of cysteine, its isolation, if present in the products of protein hydrolysis, presents difficulties which have as yet not been mastered and the occurrence of cysteine as a primary unit of protein structure is problematical.

Ergothioneine, isolated by Tanret from ergot in 1909 and identified as a constituent of blood in 1927, is one of the two betaines known to occur in the blood and tissues of the mammalian organism. Its origin from a sulfhydryl derivative of histidine, thiolhistidine, has been suggested. Thiolhistidine has recently been synthesized but attempts to prepare its methylated derivative have not been equally successful. The biological significance of ergothioneine is not known. Its presence in the blood, however, contributes to errors in those quantitative determinations which involve reductions, since its sulfhydryl group reacts similarly to that of glutathione.

The isolation and characterization of glutathione by Hopkins in 1921 is usually considered one of the important biochemical discoveries of recent years. Although the structure proposed was later shown to be incorrect by Kendall and by Hopkins himself, the discovery has influenced im-

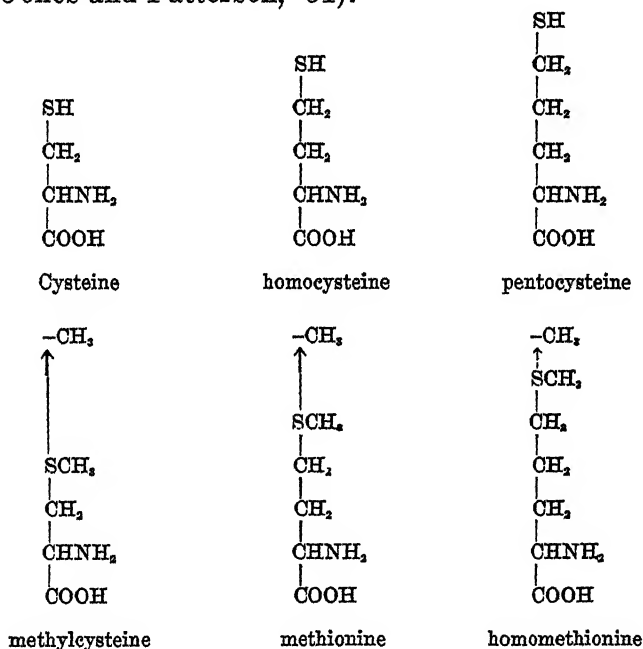
portantly the recent studies of biological oxidations and reductions. Many of the investigations concerning its distribution in tissues, and the attempts to correlate distribution and function are invalidated by the inadequate and non-specific methods used for its determination. To cite one example only, the ubiquitous occurrence of ascorbic acid, which reacts similarly to glutathione in the iodine titration method most commonly used for the estimation of glutathione, has robbed much of the early work of its significance. A recent writer (Gibson, '34) has even doubted the generally accepted structure for glutathione and has minimized its importance biologically. "Nine years ago glutathione had been isolated and 'synthesized.' Today its constitution is far from decided and it seems that the purer it is obtained, the less does it behave in vitro as its biological significance would make us expect."

An acceptable synthesis of glutathione is urgently needed to establish its structure and a specific method for its determination will make possible significant advances in our knowledge of the distribution, origin, and function of this tripeptide.

With the successful crystallization of insulin in 1926 by Abel and his co-workers, the more exact knowledge of its chemistry has been made possible. That it is a protein or a derivative of a protein, and that for its proper functioning, the sulfur-containing component, probably cystine, is essential, seems definitely established by the work of Jensen, du Vigneaud and others (Jensen and Evans, '34).

The detoxication of certain benzene derivatives, notably the monohalogen substitution products, by the animal organism results in the formation of one of the most interesting and complex sulfur-containing compounds of which we have knowledge, bromophenylmercapturic acid. The synthesis of the mercapturic acids involves the reduction of cystine to cysteine, the acetylation of the amino group and the union of the benzene nucleus with the sulfhydryl group of cysteine. The effectiveness of this detoxication seems to be dependent largely upon the available supply of cystine (White and Lewis, '32).

In addition to these compounds of sulfur known to exist and function in the living organism, certain others, chemically related to these, have been prepared and studied in their biological relationships in the belief that information as to the metabolism of the naturally occurring sulfur compounds might result. Of this group, the homologues of cysteine (or cystine) and methionine are of greatest interest. Butz and du Vigneaud ('32) observed that when methionine was heated with sulfuric acid for some hours, demethylation occurred and the next higher homologue of cystine, which they designated as homocystine, could be isolated. The next higher member of this homologous series, pentocystine, and the next higher homologue of methionine, homomethionine, which bears the same relation to pentocystine as methionine to homocystine, have recently been synthesized (du Vigneaud, Dyer, Jones and Patterson, '34).

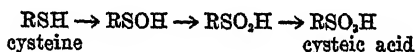


As already mentioned, thiolhistidine, the probable precursor of ergothioneine and important in that a sulfhydryl



group is here attached to a ring structure, has been synthesized. Although the attempts to demonstrate the presence of this amino acid in the protein molecule are suggestive, definite proof of its existence as a building stone of proteins is still lacking.

It is known that oxidation of cystine by such agents as hydrogen peroxide or nitrous acid gives rise to sulfuric acid. Since in the animal body, the oxidation of cystine also leads to the formation of sulfuric acid, the study of the in vitro oxidation of cystine is important. It has long been known that the oxidation of cystine to the sulfonic acid, cysteic acid, occurs readily but the resistance of this substance to further oxidation makes it improbable that the biological oxidation of cystine to sulfates follows this path. Toennies and his associates ('34) have proposed a 'stepwise' oxidation of cystine and cysteine and have been successful in the synthesis of some sub-oxidation products of cystine and cysteine. The formulae of the products of partial oxidation of cysteine are here presented.



While the exact significance of these compounds is yet to be demonstrated, preliminary results of their study biologically are promising (Hammett, '33).

Mention should also be made of existence of the thiocyanate ion in small amounts in blood, saliva and other secretions, and urine. Neither the significance nor the origin of this sulfur compound is known.

Of the reactions for the qualitative detection of the biologically important sulfur compounds, the nitroprusside-ammonia reaction has perhaps the greatest interest. This reaction of sulfhydryl compounds with sodium nitroprusside in the presence of ammonia to yield a purple-red complex, first described by Gola in 1902, was little used, until Arnold in 1907 and Heffter in 1908 called attention to its significance. Since glutathione is present in tissues mainly in the reduced or sulfhydryl form, this reaction has been employed extensively as an index of its distribution and also served as an

outside indicator in the iodimetric titration of glutathione in the Tunncliffe method. In the interpretation of data obtained by the application of this reaction to tissues or fluids, it must be borne in mind that the presence of other sulfhydryl compounds, as cysteine or ergothioneine, has not been excluded. A modification of this reaction, the cyanide-nitroprusside test, has been described for the detection of cystine. Cystine is first reduced to cysteine by sodium cyanide and the color of the nitroprusside complex is obtained on the addition of the nitroprusside. It must be remembered that the cyanide-nitroprusside reaction will be positive in the presence of any sulfhydryl compound or of any compound which by reduction yields a sulfhydryl group.

TABLE 2

*Chemical methods used for the differentiation and determination of the biologically important sulfur compounds*

1. Nitroprusside—ammonia reaction. SULFHYDRYL.
2. Cyanide—nitroprusside reaction. DISULFIDE.
3. Non-specific cystine methods. (These determine any SH group, free or formed by reduction of SS groups.)
  - a. Colorimetric. Folin methods (reduction of phosphotungstic complex).
  - b. Iodimetric. Okuda (oxidation of SH by  $I_2$ ).
  - c. Gravimetric. Vickery-White (precipitation of  $Cu^+$  complex of sulfhydryl and gravimetric determination of S).
4. Specific cystine methods.
  - a. Naphthoquinone-sulfonic acid reaction. Sullivan.
5. Methionine.
  - a. Determination of methyl groups removable by HI. Baernstein.

The older methods for the determination of cystine were gravimetric; the cystine was precipitated from a protein hydrolysate or biological fluid and either weighed or the value calculated from the determination of the sulfur content of the crude precipitate of cystine. Although cystine is insoluble in pure solutions at its isoelectric point, the solubility is influenced by the presence of other substances, both organic and inorganic, so that complete precipitation from a protein hydrolysate or from urine is difficult to secure.

Four methods or modifications of these have been most commonly used for the determination of cystine in recent years (table 2). Three of these are based upon the general

reactions of the sulfhydryl group obtained by reduction of cystine with cyanide, sulfite or a metal and hydrochloric acid. These reactions are not entirely specific for the presence of cystine. The fourth, the naphtho-quinone-sulfonic acid method of Sullivan, is the only one which seems to possess a high degree of specificity. Of the non-specific methods, the iodimetric titration procedure of Okuda gives results most nearly comparable to those obtained by the Sullivan method. The Folin-Marenzi determination, which has the advantage of simplicity, undoubtedly gives values which are somewhat high due to the reaction of substances other than cystine or cysteine with the reagent. The recently published method of Vickery and White has not been used extensively, so that it is difficult to judge its value.

For the determination of methionine, only one method, that of Baernstein ('32 a), is as yet available. The validity of the hypothesis, upon which this method is based, that methionine alone of the constituents of the protein molecule yields methyl iodide on treatment with hydroiodic acid, must be established. In view of the demonstrated interrelationship between cystine and methionine in metabolism, it is desirable to have available complete and accurate information concerning the quantitative relationships of these amino acids in proteins. A satisfactory method for the determination of methionine in tissues of blood, urine, and other body fluids is much needed.

When protein is ingested by a normal individual, its sulfur is eliminated in oxidized form by the kidney. The chief fraction of the total sulfur eliminated is the inorganic sulfate sulfur fraction, precipitable by barium chloride without previous acid hydrolysis of the urine. In addition, a small portion of the oxidized sulfur is combined in ester linkage with certain aromatic or similar cyclic substances, the so-called ethereal or conjugated sulfuric acids, sulfur which is not precipitated by barium chloride unless the ester linkage has been split by acid hydrolysis. In addition there are normally present, in small amounts, sulfur compounds of organic nature, usually grouped as the 'neutral' or organic sulfur of the urine

(Amann, '33). A high level of protein in the diet results in a marked increase in the excretion of oxidized sulfur, but not of the organic sulfur. This indicates a ready and complete oxidation of the sulfur-containing amino acids of the protein molecule. When the amino acids themselves are ingested, a similar ready oxidation to sulfuric acid occurs provided the amounts fed are not greatly in excess of the physiological limits. The relative constancy of the organic sulfur excretion and its independence of the level of general protein metabolism are best shown by Folin's classical experiments (table 3).

TABLE 3

*The influence of the protein of the diet on the distribution of urinary sulfur in man (Folin, '05)*

	<i>High protein, gm.</i>	<i>Low protein, gm.</i>
Total nitrogen	16.8	3.6
Total sulfur	1.456	0.304
Inorganic sulfate sulfur	1.308 (90%)	0.184 (60%)
Conjugated sulfate sulfur	0.076 (5%)	0.040 (13%)
Organic sulfur	0.072 (5%)	0.080 (26%)
N: S	11.5	11.8

It is assumed that, since the sulfur of cystine or methionine is so readily oxidized by the animal organism, any intermediary product of this metabolism will also be readily oxidized on administration. This method for the study of cystine or methionine derivatives has proven of much value. While ready oxidation of the sulfur does not necessarily prove a physiological function, failure of oxidation can certainly be considered to indicate lack of any important physiological relationship. This may be illustrated by a consideration of cysteic acid. Cysteic acid may be easily prepared from cystine by oxidation. It has been postulated that the physiological path of oxidation of cystine is through cysteic acid and its derivative, taurine. However, neither of these substances administered to the usual laboratory animals increases the excretion of oxidized sulfur, indicating a failure of complete oxidation of the sulfur of these compounds. This argues strongly against the formation of cysteic acid as a

normal intermediary product of cystine metabolism. We have recently synthesized and studied in our laboratory peptides containing these two sulfonic acid derivatives (White, J., '33). It seemed possible that, if cysteic acid were formed in the oxidation of cystine, the preliminary oxidation might occur while the cystine was still in peptide form and that the sulfur of peptides containing taurine or cysteic acid might be oxidized. A parallel is found in the failure of oxidation of the pyrimidine ring when the free pyrimidine bases are fed, and the ready oxidation of the same bases when they are administered in a combination existing in the tissues, that is, as nucleosides, nucleotides, or nucleic acids. In the case of the sulfonic acid peptides, Mrs. White has been unable to secure evidence of any similar relationship; no oxidation of the sulfur could be demonstrated.

Attempts have been made to approach the problem of the reactions involved in the oxidation of cystine by 'blocking' certain reactive groups of the molecule and investigating the biological behavior of these derivatives. When one hydrogen of the  $\alpha$ -amino group of an amino acid is replaced by an acyl or similar group, the resulting compound is not readily deaminized. It is usually believed that deamination is a primary step in the catabolism of amino acids. When normal deamination of cystine is prevented by 'blocking' the amino group of cystine by combination with a phenylureido or benzoyl group, the oxidation of the sulfur does not occur normally and the substituted derivative is excreted, in large part unchanged in the urine. In view of the fact that in the chemical laboratory, the sulfur of cystine is readily oxidized without affecting the  $\alpha$ -amino group, it is difficult to explain this failure of oxidation in vivo when the  $\alpha$ -amino group is 'blocked.' Similar experiments with S-substituted derivatives of cysteine, as S-benzyl- or S-ethyleysteine, have indicated that a second condition for ready oxidation of the sulfur is a free sulfhydryl group. It appears that for the oxidation of the cystine the most effective point of attack is the primary amine group and the least effective, the carboxyl, with the

disulfide or sulfhydryl group occupying an intermediate position. Despite much research, we still lack experimental data, adequate to explain the mechanism of oxidation of the sulfur of cystine. Whether study of the recently synthesized 'sub-oxidized' derivatives of cystine and cysteine of Toennies and his co-workers ('34) will afford the necessary evidence remains to be determined.

It is possible that these studies of the oxidation of the sulfur of cystine may throw some light on the mechanism of the synthesis of taurocholic acid of the bile. Garrod has estimated that 30 per cent of the total cystine metabolized is concerned in the synthesis of taurine. It is difficult to understand how accurate data for such an estimate can be secured in view of the lack of adequate information as to the volume of bile normally secreted and the paucity of satisfactory analyses of bile. It seems probable to the writer that in the synthesis of taurocholic acid, the cholic acid is first conjugated with cystine or some as yet unidentified intermediary product and that the product of conjugation is then further oxidized to taurocholic acid. If this hypothesis is correct, free taurine would not occur in the higher organisms and its formation from cystine would result from a deviation of the normal metabolic path. The facts that the amino group is not free in taurocholic acid and that cystine is not oxidized normally when the amino group is 'blocked' suggest strongly that the preliminary 'blocking' of the amino group of cystine by cholic acid results in a failure of the usual oxidative mechanism and leads to oxidation of the sulfhydryl to a sulfonic acid derivative. It must be admitted that experimental proof of this hypothesis is as yet lacking; on the other hand, no satisfactory evidence of direct synthesis from cysteic acid or taurine and cholic acid is available. It must be remembered, however, that free taurine is found in the tissues of certain invertebrates, notably the abalone.

The necessity of the free amino group for the normal metabolism of the sulfur of cystine finds a parallel in the metabolism of certain heterotrophic bacteria (Tarr, '34).

Hydrogen sulfide formation from cysteine by *Proteus vulgaris* is dependent upon the presence of a free amino group. Thus hydrogen sulfide is readily formed from cysteine, less readily from homocystine, but not from the product of reductive deamination of cysteine,  $\beta$ -thiolpropionic acid.

Valuable information concerning the metabolic interrelationships of the sulfur-containing amino acids may be obtained from growth experiments carried out with white rats on a diet deficient in cystine. In their classical experiments, Osborne and Mendel demonstrated that cystine was the first factor limiting growth in rats on a low protein diet, deficient in cystine, and that without altering the level of protein, normal growth could be obtained by the addition of cystine to the diet. These observations form the basis of many important investigations. If some other compound containing sulfur, when added as a supplement to the cystine-deficient diet, leads to an acceleration in the rate of growth, the assumption is made that the compound in question is interchangeable with cystine nutritionally or that it is a product of the normal catabolic reaction of cystine which may or may not be reversible. Thus neither elementary sulfur nor inorganic sulfates can serve as effective supplements to a cystine-deficient diet, indicating the necessity of sulfur in organic combination for the requirements of the higher forms. Cysteine supplements a cystine-deficient diet as effectively as cystine (Mitchell, '31), a finding in harmony with the usually accepted belief that the reaction  $\text{cystine} \rightleftharpoons \text{cysteine}$  is easily reversible in vivo as well as in vitro. Neither taurine nor cysteic acid, oxidation products of cystine, can replace cystine in the diet. Of particular interest also is the failure of the deamination product of cystine, the disulfide of  $\alpha$ -hydroxy  $\beta$ -thiolpropionic acid, to replace cystine. In summary, it may be stated that with the possible exception of the decarboxylation product of cystine, cystinamine, concerning which the data are conflicting (Sullivan, Hess and Sebell, '31; Block and Jackson, '32), no derivative of cystine is known to replace cystine nutritionally in experiments with rats.

In view of the many negative results obtained with cystine derivatives, it is particularly significant that methionine has been observed to supplement a cystine-deficient diet as effectively as cystine (Jackson and Block, '32). This finding explains the peculiar results of Sherman and Woods ('25), who in biological assays of casein state that casein biologically was equivalent to a content of 1.3 to 2.5 per cent of "cystine (or cystine plus nutritionally equivalent sulfur containing radicles)." This result was difficult to explain in view of the fact that chemical analyses of casein showed a content of cystine of approximately 0.3 per cent. Since it is now known that casein contains a relatively high per cent of methionine (3.25 to 3.53 per cent) according to Baernstein ('32), it is possible to interpret the experiments of Sherman and Woods on the basis of the interchangeability of cystine and methionine. Methionine is the 'nutritionally equivalent sulfur-containing compound.' Sherman appears to have been the first to suggest the existence of a substance in proteins which functions similarly to cystine in nutrition. Although methionine had been isolated at the time of Sherman's studies, neither its chemical structure nor its wide-spread distribution in protein was known. Methionine has also been shown to influence the detoxication and conjugation of bromobenzene and the synthesis of the mercapturic acids in both dogs (White and Lewis, '32) and white rats (White and Jackson, '33) in a manner similar to cystine. Further evidence of interrelationship of cystine and methionine is to be obtained from studies of cystinuria to be discussed later.

The demonstration of the similar functions of cystine and methionine led to a more careful study of the metabolism of the more recently discovered of the two sulfur-containing amino acids. The sulfur of methionine, like that of cystine, is readily and completely oxidized to sulfates when methionine is administered to man or the common laboratory animals (Virtue and Lewis, '34; du Vigneaud, Loring and Craft, '34). It also appears that, when deamination is prevented by 'blocking' the amino group, the N-substituted derivatives of methionine, as well as those of cystine, are not



oxidized normally, although the evidence concerning this point is meagre (Virtue and Lewis, '34).

In experiments with rats and rabbits, the administration of methionine has been followed by the excretion of a substance which gives the reactions of a compound containing the disulfide linkage (SS) but fails to give the more highly specific Sullivan test for cystine (Chase and Lewis, '33; Virtue and Lewis, '34). In view of the fact that demethylation is not an uncommon biological reaction, and that demethylation of methionine has been demonstrated to occur readily *in vitro* by Butz and du Vigneaud ('33), the presence of the demethylation product of methionine, homocystine, or its disulfide, homocystine, in the urine is suggested. Further support of the theory of demethylation as a reaction in the intermediary metabolism of methionine is afforded by the recent observations of Pirie ('34) that when slices of liver tissue of rats and methionine solutions are incubated together, the fluid gives, in all cases, a faint but definite nitroprusside reaction. The isolation of homocystine from the urine or other biological fluids is necessary to furnish the final proof of this hypothesis, at present supported by indirect evidence only.

The problem has been further clarified by the recent studies of homocystine as a supplement to a cystine-deficient diet (du Vigneaud, Dyer and Harmon, '33). The promotion of growth of young white rats under these conditions is as effective as when methionine or cystine is added as a supplement. Thus we are able to state with some certainty that these three amino acids are equivalent nutritionally, so far as growth is concerned.

It might be expected that demethylation is the normal path of metabolism of all the homologues of methionine. The sulfur of methionine, homocystine, and S-methylcystine is oxidized readily in the organism of the rabbit (du Vigneaud, Loring, and Craft, '34). Demethylation does not appear to be the primary reaction of the catabolism of S-methylcystine, however, since this compound is not an effective supplement of a cystine-deficient diet (du Vigneaud, Loring,

and Craft, '34). If demethylation were to occur prior to other changes in the molecule, cysteine or cystine should result and S-methylcysteine would be expected to serve as a source of cystine for growth. If the role of methionine as a substitute for cystine in growth experiments is to be explained by its conversion to homocysteine, the negative results with S-methylcysteine would indicate that the reactions of metabolism of the members of the homologous series of methylthiol amino acid are not similar.

Neither homomethionine nor the oxidized form of its demethylation product, pentocystine, function as do cystine, homocysteine and methionine in promoting growth in rats on a diet low in its content of cystine (Dyer and du Vigneaud, '35). This indicates that the peculiar nutritional role of the three sulfur-containing amino acids which appear to be interchangeable in growth promotion is not simply a function of the disulfide (SS) or sulfhydryl (SH) group alone, but is related to the structure of the molecule as a whole. Only the sulfur homologues containing an  $\alpha$ -amino group and three or four carbon atoms appear to possess significant biological properties.

Further evidence in support of the biological relationship of cystine and methionine is to be obtained from recent studies of the interesting 'error' of metabolism, cystinuria. For many years it has been known that the cystinuric individual, while excreting considerable quantities of cystine particularly when on a high protein diet, was able to oxidize completely the amino acid itself, when fed. No satisfactory explanation of this anomaly was presented. Brand and co-workers ('35) have shown that the explanation presumably is to be found in the fact that the methionine and not the cystine of the diet is the source of the cystine excreted in cystinuria. Administration of cystine to a cystinuric failed to increase the cystine excretion while administration of methionine led to a marked increase in cystine. We have been able to confirm these findings with a cystinuric patient in our own laboratory. Brand has also observed that cysteine augments the

cystine excretion. He postulates a direct chemical conversion of methionine to cysteine, which is difficult to picture in the light of any known mechanism of chemical transformation in the organism, and a path of metabolism of cysteine distinct from that of cystine. Homocystine was oxidized readily and completely by the cystinuric, while the administration of homocystine led to a significant augmentation of the cystine content of the urine.

Despite the many advances in recent years, the main problem of the function of cystine remains unanswered. What is the function of the sulfur? Is the equilibrium  $\text{cysteine} \rightleftharpoons \text{cystine}$  as easily reversible in vivo as is usually assumed? Is the function of cystine related to the SS or SH linkage? The fact that homocystine is equivalent to cystine in nutrition might suggest this. On the other hand, it will be recalled that the  $\alpha$ -hydroxy-derivative of cystine, the so-called 'des-aminocystine,' which differs from cystine only in the absence of the  $\alpha$ -amino group, cannot replace cystine, a fact which speaks against the function of the SS group alone.

Are we to postulate an actual synthesis of cystine from methionine through homocystine, or homocysteine, or is there an intermediary product common to both these amino acids through which they function in metabolism? When methionine is fed to a dog receiving bromobenzene, is there synthesized a new, as yet unknown, conjugation product, a mercapturic acid of which acetylhomocysteine is a constituent rather than the usual acetylcysteine, or does the methionine when administered take over some necessary metabolic function of the cystine, thereby making available cystine for mercapturic acid synthesis? If demethylation is the primary reaction of the intermediary metabolism of methionine, is the methyl group liberated made available for purposes of synthesis, as for example, the methylation of glycine as a stage in the synthesis of creatine? What is the relation of cystine or methionine to insulin? To glutathione? What is the function of the latter? These are some of the problems of sulfur metabolism, the solution of which is essential before

a coherent picture of the relation of the sulfur compounds to each other and to the function of the living organism can be presented.

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# A MODIFICATION OF THE SHERMAN METHOD OF STUDYING THE MULTIPLE NATURE OF VITAMINS, WITH AN APPLICATION TO VITAMIN G

T. S. HAMILTON AND H. H. MITCHELL

*Division of Animal Nutrition, University of Illinois, Urbana*

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Since the vitamin B complex was definitely shown to consist of at least two separate and distinct components, now referred to as vitamin B or B<sub>1</sub> and vitamin G or B<sub>2</sub>, much evidence has been presented in the literature that each of these components is divisible into two or more entities. Reviewers of this work have often hesitated to assign to the evidence for some particular unknown factor any definitive significance, although admitting the belief that in all probability the old B complex consists of more than vitamins B and G.

The difficulties in the way of according full significance to such evidence are, first, that potent concentrates of the presumably unknown factors have so seldom been prepared, and, second, that the evidence of the existence of new factors does not consist of the production of characteristic symptoms of malnutrition by their removal from the diet, but merely of certain more or less well-defined and reproducible disturbances of growth, such as a 'plateau' in the curve of growth. It is perhaps too seldom realized that failure of growth is a non-specific phenomenon, in itself affording no indication of its etiology, and in particular that such failure may be the result of a quantitative dietary deficiency as well as of a qualitative deficiency. Also, the fact that a different amount

of a given vitamin concentrate is required to maintain weight or growth than to prevent or cure specific symptoms of vitamin deficiency is not valid evidence of the multiple nature of a vitamin, since Baumann, Riising and Steenbock ('34) have shown the same to be true of chemically pure carotene.

An ingenious method of testing the possible multiple nature of a given vitamin was applied by Sherman and Axtmayer ('27) to the old B complex, and later in the same laboratory by Stiebeling ('31-'32) and by Page ('34) to vitamin G(B<sub>2</sub>). The method consists of fractionating a selected good source of the vitamin under study by some likely chemical procedure. The two fractions are then tested separately and in equivalent amounts as supplements to a basal G-free diet, and in a third experiment they are tested in combination, using as daily supplements exactly one-half of the amounts of each tested alone. If the vitamin under test consists of two (or more) distinct factors, it would be unlikely that they would be similarly distributed between the two fractions of the vitamin containing material. If they have been partially, if only incompletely, separated by the method of fractionation selected, then the combination of the two fractions may induce a more rapid and a more sustained growth than either of the fractions by itself. If the superiority of the combination of fractions is statistically demonstrable, then a supplementary rather than an additive relation between the fractions has been established, and the conclusion has been justified that the vitamin under study possesses a multiple nature. But if the combination is no better than one of its fractions (in double amount), or particularly if it is midway in value between both, the interpretation is complicated. However, such indecisive results afford no evidence that the vitamin is a single entity.

Sherman and Axtmayer emphasized their use of a 'more quantitative method' of attacking their problem "to the end that the results thus obtained might be more conclusive as to the multiple nature of vitamin B." Their results were in line with those of a number of previous studies in indicating

the complexity of the old vitamin B, and both Stiebeling and Page have submitted evidence of like significance for the multiple character of vitamin G.

This method of Sherman is quantitative to the extent that the intake of vitamin supplements is quantitatively controlled, but it is not quantitative with reference to the intake of the basal diet, containing all other nutrients, since the basal diet is fed *ad libitum*. The amount of it voluntarily consumed by the experimental rats is neither reported nor in any way considered in the interpretation of the results.

The advantages of controlling the intake of basal diet as well as of the test supplements are real and demonstrable. A variable intake of basal diet with a constant intake of supplement will demonstrably contribute to the variability of the gains in weight secured. Hence, the precision of the average gain secured would be increased if the intake of basal diet as well as of test supplement is equalized. When two qualitatively different supplements are being compared with reference to their growth-promoting power, another source of confusion is introduced if the two supplements possess a differential effect upon appetite, because this differential effect will induce a differential consumption of the basal diet. It is true that the more potent growth-promoting supplement will in general stimulate appetite to the greater extent, but this is not true without exception, and when not true the average gains in weight induced by the two supplements may give a false conception of their relative inherent values in correcting the nutritive deficiency of the basal diet.

If the appetite stimulation of the test supplements parallels their growth-promoting powers, equalization of the intake of the basal diet by the experimental animals, preventing any expression of the appetite effect, will not obscure the growth effect, but will identify it as such beyond question. The separate observation and measurement of the various factors influencing animal growth simplifies the interpretation of the results obtained, if it is not essential, in all cases, to any definite interpretation. If a test supplement exerts an effect



only upon appetite, its dispensability as a participant in animal metabolism can only be proved by complete control of the food intake of experimental animals.

The greater technical precision attainable in vitamin tests by the complete control of the food intake of experimental animals was demonstrated by Garrett and Mitchell ('33). In an application of the paired-feeding technic to vitamin A assay, they were able to show statistically<sup>1</sup> for their particular basal diet, and using eight pairs of rats in testing each ration, that an inclusion of 4.0 per cent or less of a sample of butter-fat provided inadequate amounts of vitamin A for maximum growth, while an inclusion of 4.5 per cent or more provided adequate amounts. The method is thus capable of detecting a difference in growth-promoting value of rations containing no more than a 12 per cent difference in vitamin A content. Smaller differences were not tested. With their method of uncontrolled feeding of the basal diet, Sherman and Batchelder ('31), on the basis of questionable 'interpolations' between experimental observations, claim only that "a decrease of 25 per cent (or an increase of 33 per cent) is undoubtedly measurable," while Coward's ('33) results would indicate an even less precise decision.

An improvement in the precision of the Sherman method for detecting the multiple nature of a vitamin would appear to be to make the comparisons of the growth promoting value of the fractionated supplements (2a, 2b, a + b) according to the paired feeding technic, pair mates to receive the same amount of basal diet but different supplements.

This presumably improved technic was applied to the problem attacked by Page ('34) relative to the multiple nature of vitamin G. The procedure of Page was followed in all details, with few exceptions other than the method of feeding. The food source of vitamin G was dried skim milk powder, and the two fractions were prepared, as directed in Page's paper, by extracting the powder with 93 to 94 per cent ethyl

<sup>1</sup> Assuming that a probability of 0.05 or less that the outcome of a comparison is a chance result may be neglected.

alcohol (by weight), concentrating the extract to a small volume, and precipitating with an equal volume of ethyl ether. The two fractions tested for supplementing value were 1) the extracted skim milk powder, and 2) the ether precipitate. The basal diet was the vitamin G-free diet of Bourquin and Sherman. Feeding the basal diet *ad libitum* to rats that survived a depletion period of 56 days, Page observed (in groups of four animals each) that 1) a daily supplement of 0.3 gm. of the alcohol-extracted milk powder merely maintained the animals at practically constant weight throughout the feeding period of 5 weeks, 2) a daily supplement of 0.07 gm. of the ether precipitate induced an average weekly gain of  $6.4 \pm 0.3$  gm., and 3) a daily supplement of 0.15 gm. of the alcohol-extracted milk powder and 0.035 gm. of the ether precipitate induced an average weekly gain of  $5.7 \pm 0.4$  gm., not statistically to be distinguished from the gain on 0.07 gm. of the ether precipitate. Page interprets these results as follows:

In the present stage of our knowledge, the only satisfactory explanation of this supplementary relation between the 2 fractions would seem to be that a long depletion period (56 days) results in depleting the bodily reserves of the rats of a second limiting factor. The first limiting factor is carried by both the ether precipitate and the alcohol extracted milk powder, the second factor by the ether precipitate only. This interpretation is applied only as a suggestive hypothesis which best fits the experimental facts so far available.

However, it would appear that the evidence as thus presented for the existence of two factors in vitamin G is to some extent indecisive, since the results may be explained on the assumption, which cannot be controverted from the data, however improbable it may be, that 0.3 gm. of the extracted milk powder contains a small and inadequate amount of G, but that 0.035 gm. of the ether precipitate contains an adequate amount of G. Hence in order to prove beyond dispute the multiple nature of G, the combination of fractions should be superior in growth promoting value to either fraction when fed separately in double amount.

In repeating Page's study, his directions were followed faithfully with the following exceptions: The paired feeding technic was used in place of *ad libitum* offering of the basal diet. The same amounts of the same supplements were used, but the caloric value of the supplements was equalized as closely as possible by giving daily as much extracted corn starch as was needed to bring the dry matter of the daily supplements in all cases to 0.3 gm., the size of the largest supplement, i.e., the extracted milk powder. No coprophagy harness was used; however, the rats were kept upon  $\frac{1}{2}$  inch mesh wire screening. To maintain the appetite of the rats and to prevent the occasional appearance of neuritic symptoms, it was found necessary to give to each rat 1 drop of tiki-tiki extract twice a week. This necessary addition, indicating that the basal diet did not contain an adequate concentration of vitamin B in spite of its content of an alcoholic extract of wheat, was disturbing, but would not vitiate the results unless the small amounts of tiki-tiki extract consumed contained a G component in adequate amounts for maximum growth. Thus supplemented, the diet permitted the development of growth promoting distinctions between preparations made from milk powder that would contain little if any vitamin B, because of the much greater solubility of B than of G in strong alcohol, and because of the solubility of vitamin B in ethyl ether (Cooper, '12; Block and Cowgill, '32).

The results of the experiment, which was continued for 5 weeks, are summarized in table 1. It is clear from this summary that no evidence was obtained that the alcohol extracted milk powder and the ether precipitate of the alcohol extract supplemented each other as sources of G potency. The ether precipitate was distinctly superior in growth promoting value to the alcohol extracted milk powder, and was probably superior to the combination of one-half portions of each. This combination, in turn, was superior to the alcohol extracted milk powder. The results are fully explained on the supposition that the basal diet is deficient in only one indispensable factor, vitamin G, present in the ether precipitate in

TABLE 1  
*Statistics of the paired feeding comparison of the growth promoting value of alcohol extracted skim milk powder (A) and the ether precipitate of the alcohol extract (B) as supplements to a vitamin G-free diet*

SUPPLEMENTS FED DAILY		NUMBER OF PAIRS OF RATS	AVERAGE DAILY INTAKE OF BASAL DIET	NUMBER OF FOOD REFUSALS ON		AVERAGE TOTAL GAIN ON		DIFFERENCES IN TOTAL GAIN BETWEEN PAIR MATES			DIFFERENCES IN BODY LENGTH BETWEEN PAIR MATES		
Number 1	Number 2			Suppl. 1	Suppl. 2	Suppl. 1	Suppl. 2	Mean <sup>1</sup>	Standard deviation	Probability <sup>2</sup>	Mean <sup>1</sup>	Standard deviation	Probability <sup>2</sup>
0.3 gm. of A	0.07 gm. of B	6	3.73 gm.	164	44	7.3 gm.	12.3 gm.	+ 5.00 gm.	2.31 gm.	0.0024	+ 3.67 mm.	2.49 mm.	0.011
0.3 gm. of A	0.15 gm. of A 0.035 gm. of B	6	3.88	90	64	10.5	14.7	+ 4.17	2.19	0.0041	+ 0.67	1.79	0.23
0.07 gm. of B	0.15 gm. of A 0.035 gm. of B	8	3.87	48	143	22.9	19.5	- 3.37	4.95	0.058	- 1.50	6.34	0.22

<sup>1</sup> The positive sign indicates a greater gain on supplement no. 2. A negative sign, the reverse.

<sup>2</sup> Probability that fortuitous factors alone determined the differences between pair mates. If the probability is 0.05 or less, according to R. A. Fisher ('28, p. 45), it may reasonably be neglected.

considerably greater concentration than in the alcohol extracted residue. The probabilities given in table 1 (and also those in table 2) were computed from the table of 'Student' ('08).

The experiment possesses a negative significance. It seemed profitable and important to determine whether the method is capable of producing positive results where positive results are to be expected from available evidence. One of the experiments of Sherman and Axtmayer was therefore repeated by the revised method above illustrated. In this experiment a basal ration free of the old vitamin B complex was supplemented, first, with daily portions of 0.8 gm. of ground whole wheat, second, with daily portions of 0.8 gm. of autoclaved yeast, and third, with a combination of 0.4 gm. of each. With uncontrolled feeding of the basal diet, Sherman and Axtmayer were able to demonstrate a superior growth promoting value of the combined supplements to that of either supplement fed in double amount. This outcome constitutes additional evidence that the old B complex is multiple in nature.

This experiment was repeated by the revised fully quantitative technic, except that a comparison of the wheat and autoclaved yeast supplements with one another was omitted as unnecessary. In all other respects the experiment was patterned after that of Sherman and Axtmayer. However, the gains are computed, not on the basis of the final live weights, but on the basis of the final empty weights, after removal of the intestinal contents. These gains in weight are presumably more representative of the actual gains in body tissue. The results obtained are summarized in table 2. It is evident that the combination of wheat and autoclaved yeast was demonstrably superior to either supplement alone in double quantity. This constitutes conclusive evidence, both of the multiple nature of the old B complex, and of the reliability of the feeding method employed in developing positive evidence where the facts warrant.

The greater precision (accuracy) of the paired feeding technic, involving control of the intake of basal ration as well

TABLE 2

*Statistics of the paired feeding comparison of the growth promoting value of ground whole wheat (A) and of autoclaved yeast (B) as supplements to a diet deficient in both vitamins B and G*

SUPPLEMENTS FED DAILY		NUMBER OF PAIRS OF RATS	AVERAGE DAILY INTAKE OF BASAL DIET	NUMBER OF FOOD REFUSALS ON		AVERAGE TOTAL GAIN ON		DIFFERENCES IN TOTAL GAIN BETWEEN PAIR MATES			DIFFERENCES IN BODY LENGTH BETWEEN PAIR MATES		
Number 1	Number 2			Suppl. 1	Suppl. 2	Suppl. 1	Suppl. 2	Mean <sup>1</sup>	Standard deviation	Probability <sup>2</sup>	Mean <sup>1</sup>	Standard deviation	Probability <sup>2</sup>
0.8 gm. of A	0.4 gm. of A 0.4 gm. of B	8	gm. 3.14	183	16	gm. 6.2	gm. 10.4	gm. + 4.25	gm. 1.39	< 0.0001	mm. + 4.37	mm. 2.29	0.0008
0.8 gm. of B	0.4 gm. of A 0.4 gm. of B	8	3.14	120	68	17.1	19.0	+ 1.87	2.31	0.035	+ 1.75	2.68	0.066

<sup>1</sup>The positive sign indicates a greater gain on supplement no. 2. A negative sign, the reverse.

<sup>2</sup>Probability that fortuitous factors alone determined the differences between pair mates. If the probability is 0.05 or less, according to R. A. Fisher ('28, p. 45), it may reasonably be neglected.

as of vitamin supplement, over that of the method of Sherman and Axtmayer, involving control of the intake of vitamin supplements only, may be assessed from the data in these five paired feeding comparisons. This may be done by computing in two ways the probability that chance alone would produce a group difference as great as or greater than that observed in each of the comparisons of two vitamin supplements. By the first method, the probability is based upon the standard deviation of the eight differences in gain between paired rats, the method followed in a 'Student' analysis. These probabilities are given in tables 1 and 2. By the second method, the probability is computed from the standard deviation of the total gains exhibited by the rats on each of the two supplements according to a method proposed by Fisher ('28, p. 107). In this method no advantage is taken of the equalization of intake of basal diet by paired rats. If this modification in vitamin assay work is effective in increasing the precision of the test, the probabilities (P) computed by the first method should be much less than the probabilities computed by the second method. In such case the proposed modification would be a refinement in technic. But if the complete control of food intake is ineffective in accomplishing its purpose, then the probabilities computed by the second method should be less than those computed by the first method. As Fisher says:

In cases in which each observation of one series corresponds in some respects to a particular observation of the second series, it is always legitimate to take the differences and test them . . . as a single sample; but it is not always desirable to do so. A more precise comparison is obtainable only if the corresponding values of the two series are positively correlated, and only if they are correlated to a sufficient extent to counterbalance the loss of precision due to basing our estimate of variance upon fewer degrees of freedom.

The values of  $P^2$  computed by the two methods for the five comparisons given in tables 1 and 2 are, in order, 0.0024 and 0.081, 0.0041 and 0.068, 0.058 and 0.11, less than 0.0001 and 0.019, 0.035 and 0.35. It is clearly evident that the precision

<sup>2</sup> In using Fisher's table of t values, the corresponding values of P should be halved to correspond to the 'Student' probabilities (see p. 105 of Fisher's book).

of the assay has been greatly improved by the equalization of the intake of basal diet between comparable animals.

Perhaps some quantitative idea of the increase in precision of the paired over the unpaired comparison of gains may be obtained from the ratio of the standard deviations of the mean difference in gain between each pair of supplements as computed, first, from the standard deviations of the gains made on each supplement, and, second, from the paired differences in gain. This ratio varied from 1.01 to 3.8 and averaged 2.3. Hence, it may be concluded that the equalization of the intake of basal diet for comparable animals increased the precision of the comparison of the growth promoting value of vitamin supplements 2.3 times.

The complete equalization of the food intake of comparable animals thus greatly improves the accuracy of vitamin assay methods involving growth measurements only. It is not unreasonable to expect that it would improve also those vitamin assay methods concerned with the prevention or the cure of deficiency disease symptoms. Clear indications to this effect are the findings of Amantea ('33) and of Westenbrink ('34), that the time of appearance of polyneuritis in pigeons is directly dependent upon the amount of vitamin B-free diet consumed, and those of Watkins and Mitchell ('35) that the more rachitogenic diet rats consume the quicker do rachitic symptoms develop.

#### CONCLUSIONS

Both the precision and the clarity of vitamin assay methods are improved by controlling the consumption by the experimental animals of the basal diet, as well as of the vitamin supplements. In particular, the Sherman and Axtmayer method of determining whether or not a given vitamin possesses a multiple nature, is rendered completely quantitative by extending the control of food intake to the basal diet. The precision of the comparison of the growth promoting value of two vitamin supplements is increased more than twice by this modification, and any ambiguity in interpretation occasioned by a differential effect upon appetite of different vita-



min fractions, whether containing single or multiple supplementing factors, is removed.

Applying this modified Sherman and Axtmayer method to two G fractions prepared from skim milk powder (according to the directions of Page), no evidence of the multiple nature of the vitamin was obtained.

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# THE INFLUENCE OF VITAMIN C LEVEL UPON RESISTANCE TO DIPHTHERIA TOXIN <sup>1</sup>

## I. CHANGES IN BODY WEIGHT AND DURATION OF LIFE

C. G. KING AND M. L. MENTEN

*Departments of Chemistry and Pathology, University of Pittsburgh, Pittsburgh*

### TWO FIGURES

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It is difficult to evaluate the physiological significance of the milder types of malnutrition, but it is reasonably certain that this phase of nutrition studies is closely related to human dietary problems. The investigations of Sherman ('32) and McCarrison ('21) have been particularly fruitful in showing the influence of deficiencies in the zone where there is no obvious manifestation of a 'deficiency disease.' A good review of the relation between malnutrition and resistance against infections has been published recently by Clausen ('34).

In the course of making repeated assays of vitamin C concentrates over a period of several years previous to its isolation and identification, it was consistently noted that there was a fairly wide zone of latent deficiency in which there was a marked loss of body tone without exterior indications of scurvy. During assays with the pure vitamin (Waugh and King, '32) from different natural sources and with the synthetic vitamin,<sup>2</sup> the same characteristic zone was

<sup>1</sup> Contribution no. 296 from the department of chemistry and from the pathology department. A summary of this paper was presented before the American Institute of Nutrition, April 10, 1935 at Detroit, Michigan.

<sup>2</sup> The synthesis was carried out through sorbitol sorbose sorbosone 2-keto-l-gulonic acid vitamin C, by Dr. E. G. White in these laboratories during 1933-1934.

observed. The finding of a high concentration of the vitamin in glandular tissue (Bessey and King, '33) together with its known intimate relation to growth, tooth and bone development, capillary strength, collagen gelation (Wolbach and Howe, '26; Menkin, Wolbach and Menkin, '33), resistance to infection (Sherman and Smith, '31), cellular respiration (Szent-Gyorgyi, '30, '31; Quastel and Wheatley, '34) and other enzyme systems (Karrer and Zehender, '33; Purr, '33, '35) gave further evidence of its probable importance in physiological processes not associated specifically with scurvy. In other words, physiological impairment from a moderate vitamin deficiency might reasonably become evident in a manner not directly associated with the usual symptoms of a complete or more drastic vitamin deficiency.

In the present investigation we have studied the resistance of guinea pigs to standardized diphtheria toxin when the animals were on different levels of vitamin C intake. The main purpose of the study was to find whether or not there is a significant lowering of resistance to the toxin in the zone of vitamin C deficiency where the typical symptoms of scurvy are not well developed (generally referred to as 'latent scurvy').

Diphtheria toxin was selected because: a) it was available as a standardized product, and b) the tissue changes induced by diphtheria toxin injections are similar in certain respects to those observed in scorbutic guinea pigs (Bessey, Menten and King, '34). It has the additional advantage of being a toxin frequently encountered in human pathology.

Rinehart and Mettier ('34) claim that there is an intimate relation between 'latent scurvy' in guinea pigs and the effect of streptococcic infections. Discordant results were found clinically, however, at the Rockefeller Institute (Schultz, Sendray and Swift, '35). During the course of the present investigation, Harde and associates ('34, '35) have reported interesting relationships between vitamin C and diphtheria toxin, with additional notations on other types of toxins arising from infections.

## EXPERIMENTAL

*Procedures.* Male guinea pigs (purchased) about 250 gm. in weight, were placed in individual cages and given the usual care observed when making vitamin C assays. During a preliminary period they were given a standard vitamin C-free (Sherman) diet (bran, oats, milk powder, butter fat, and salt, supplemented by yeast and cod liver oil) and fresh spinach, to permit them to reach an average weight of about 300 gm. and to assure a normal growth rate and apparent good health at the beginning of the experiment. They were then divided into groups whose average weights and general condition were as nearly comparable as possible. Each animal was then given a definite quantity of vitamin C daily from a graduated pipette, using standardized orange juice (2,6-dichlorophenol-indophenol titration) or a standard solution of the pure vitamin. The animals were examined practically daily and were autopsied at death or at the end of each series of tests.

Dilutions of diphtheria toxin<sup>3</sup> in sterile saline were injected subcutaneously near the groin. Because of severe edema, sloughing and occasional local infections in animals on low vitamin C levels, it was found preferable to make the injections on either side of the spinal column.

Tissue respiration measurements were made on liver and kidney tissue<sup>4</sup> from representative animals. In each instance the animals were killed by a blow over the occiput. The tissue was immediately suspended in Locke's solution without dextrose. Determinations were made on 100 mg. finely cut portions in duplicate or triplicate with a Barcroft-Warburg constant volume manometric apparatus. The oxygen consumption from air was recorded at 30-minute intervals over 2-hour periods.

Titration of vitamin C were carried out in the presence of 8 per cent trichloroacetic acid, using 2,6-dichlorophenol-indophenol as described in an earlier paper (Bessey and

<sup>3</sup> The toxin was obtained from Parke, Davis & Co.

<sup>4</sup> The respiration measurements were made by Mr. Martin Yavorsky with the apparatus at the U. S. Bureau of Mines through the courtesy of Mr. W. P. Yant.

King, '33). A carefully washed suspension of  $\text{Al}(\text{OH})_3$  was found helpful for decolorizing and clarifying extracts from liver, spleen, and kidney tissue.

#### RESULTS AND DISCUSSION

In preliminary experiments<sup>5</sup> it was found that single injections of diphtheria toxin containing 1.5 or 2.0 M.L.D. produced such extensive lesions that there was no satisfactory basis for differentiating between the effects of low and high vitamin intake. With single injections of 1.0 M.L.D., however, the survival time of five animals receiving 5 mg. of pure vitamin daily was greater (average 50 per cent) than that of four animals depleted until mild indications of scurvy were evident. In later experiments, with smaller repeated doses of toxin (0.1, 0.3 and 0.5 M.L.D.), the differences between latent scorbutic and normal animals became clearly evident, as shown in the detailed records which follow.

Degeneration with loss of lipid constituents and frequently hemorrhage of the adrenal cortices was evident as a result of either vitamin C depletion or injection of diphtheria toxin.

Titration of the vitamin C in liver, spleen, kidney and adrenal tissue of six animals receiving toxin compared to five animals on the same vitamin level without toxin did not indicate significant changes in vitamin content due to toxin injections. The titrations were made approximately 3 weeks subsequent to the injections, however, and the intermediate period might have permitted readjustments subsequent to earlier changes.

Table 1 provides a summary of the findings with thirty-eight animals in relation to survival and changes in body weight in a single experiment. Groups A and B, with fifteen animals each, received 0.50 and 0.25 mg. of vitamin C per day (given orally as orange juice), representing respectively, minimum protective and sub-protective levels. Two groups, A<sup>1</sup> and B<sup>1</sup>, with four animals each on the 0.50 and 0.25 mg.

<sup>5</sup> The authors wish to acknowledge the assistance of Dr. O. A. Bessey during the preliminary part of the work in 1933, and of Messrs. J. H. Leatham, M. Kochin and S. C. Camp in the later experiments.

levels served as controls, without toxin. For 10 days previous to the test period, all animals received 1.5 mg. per day. Animals in groups A and B were given subcutaneously 0.3 M.L.D. of toxin on the eighth, eleventh, twenty-fifth and twenty-ninth days after being put on their respective vitamin levels. Their minimum, average and maximum weights at the time of the first injection were 260, 325 and 418 gm. for group A, and for group B the corresponding values were 257, 323 and 406 gm. It will be noted that within 26 days after the first injection, the number of deaths was twice as great on the lower nutritive level (0.25 mg. per day) as on the higher level

TABLE 1

*Resistance of guinea pigs to diphtheria toxin when on different levels of vitamin C intake*

GROUP AND NUMBER OF ANIMALS	MILLIGRAMS OF VITAMIN PER DAY	TOTAL TOXIN FOUR IN- JECTIONS	NUMBER OF DEATHS WITHIN 26 DAYS	NUMBER SUR- VIVING AFTER 55 DAYS	AVERAGE SUR- VIVAL AFTER SECOND INJECTION	AVERAGE CHANGE IN WEIGHT GRAMS 55 DAYS
					<i>days</i>	
(A) 15	0.50	1.2 M.L.D.	5	3	32 <sup>1</sup>	— 54
(B) 15	0.25	1.2 M.L.D.	10	0	21	— 115 <sup>2</sup>
(A <sup>1</sup> ) 4	0.50	0	0	4	..	+ 187
(B <sup>2</sup> ) 4	0.25	0	0	4	..	— 30

<sup>1</sup> Exclusive of three animals which survived the entire period and were rapidly gaining in weight at the end of the test.

<sup>2</sup> Average net change in weight at time of death.

(0.50 mg. per day). Three animals on the 0.5 mg. level survived the entire experimental period and were gaining in weight at the end of the test. The contrast in severity and duration of subcutaneous reactions at the point of injection was striking. Animals on the higher level showed marked swelling and local congestion for several days, following which there was slow healing action. Those receiving 0.25 mg. developed more severe swelling, necrosis and hemorrhage at the site of injection. These differences were evident before there was any general indication of typical 'scurvy' among those receiving 0.25 mg. per day. At autopsy, every animal on the 0.25 mg. level showed hemorrhagic necrosis at

the site of the first two injections, while only four animals on the 0.50 mg. level showed appreciable congestion at the site of the first injection, and in these four the condition was much less severe than in the 0.25 mg. group. In view of the variations in vitamin C titration values found in tissues of individuals dying from various conditions (Yavorsky, Almaden and King, '34), these results are of interest in relation to human pathology.

A later experiment is summarized in figure 1. Two levels of vitamin intake were again compared. Thirty guinea pigs were used, with five animals in each experimental group.

After 25 days on a basal diet supplemented with spinach, the animals were divided into the six groups and given orange juice as a source of vitamin C. Those represented by curves A, B and C were then given 1.5 mg. of vitamin daily. After being depleted 5 days, those included in curves A<sup>1</sup>, B<sup>1</sup> and C<sup>1</sup> received 0.3 mg. of vitamin per day. A and A<sup>1</sup> served as control groups for the two vitamin levels, without receiving toxin injections. B and B<sup>1</sup> received 0.3 M.L.D. injections of toxin on the sixteenth, twenty-first, twenty-eighth and thirtieth days after being placed on the standard vitamin levels. C and C<sup>1</sup> received 0.5 M.L.D. injections of toxin on the same days, i.e., parallel with B and B<sup>1</sup>. The minimum, average and maximum weights of animals for the different groups at the time of the first toxin injection were as follows: A, 313, 367, 450; B, 333, 348, 360; C, 370, 395, 415; A<sup>1</sup>, 270, 302, 327; B<sup>1</sup>, 328, 361, 388; C<sup>1</sup>, 373, 413, 453.

It will be noted that the group receiving 1.5 mg. of vitamin C daily and 0.3 M.L.D. injections (curve B) suffered only a slight loss in weight, following which they resumed normal growth. When growth was resumed, the local lesions had disappeared except for a small amount of scar tissue. In contrast, those receiving only 0.3 mg. of vitamin per day, and the same amount of toxin (curve B<sup>1</sup>), were all dead at the end of the experiment and at autopsy showed severe subcutaneous hemorrhagic necrosis.

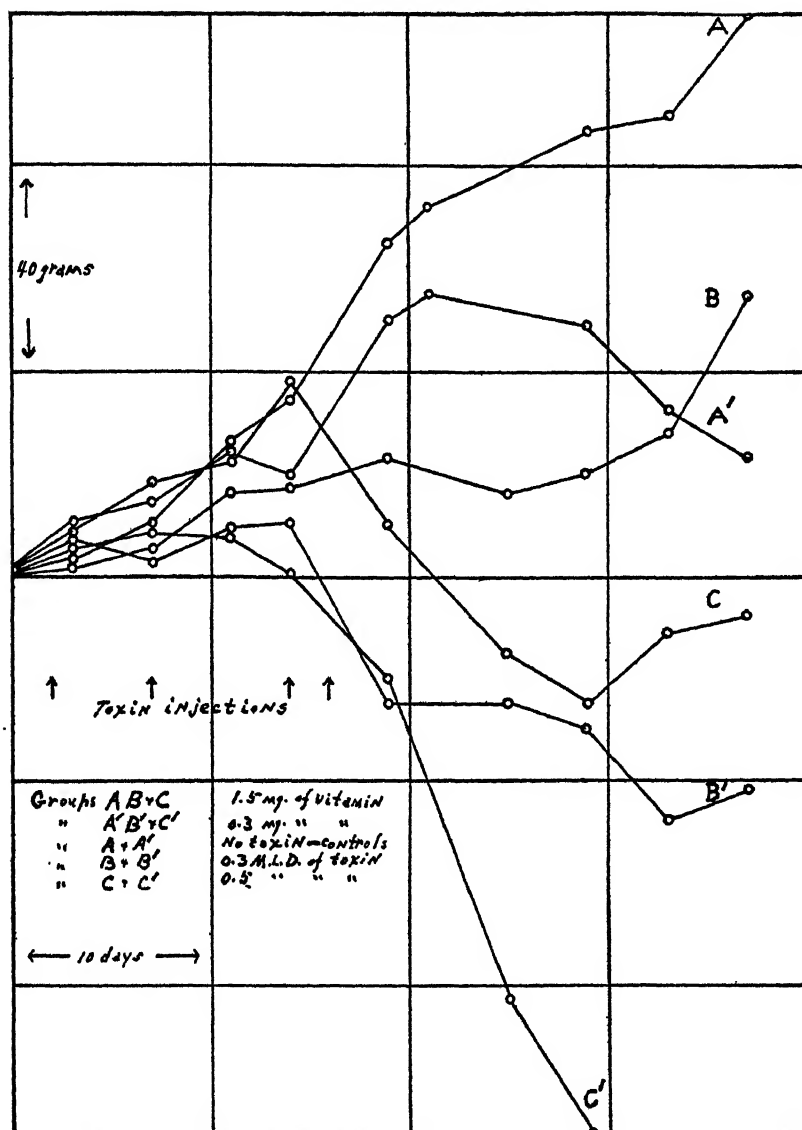


Fig. 1 Effect of vitamin C feeding level upon the resistance of guinea pigs against diphtheria toxin.



The 1.5-mg. group which received 0.5 M.L.D. injections (curve C) showed a marked loss in weight after the third and fourth injections, but two animals survived and were gaining in weight at the end of the test period. Local congestion had nearly disappeared in those surviving the test period (38 days after the first injection). In contrast to the 1.5-mg. group, the 0.3-mg. group receiving 0.5 M.L.D. injections (curve C<sup>1</sup>) began to lose weight slowly after the second injection and rapidly after the third injection. All pigs in this group showed severe local reactions and rapid loss of weight, terminating in death within 27 days after the first injection. Since the control group (curve A<sup>1</sup>) receiving 0.3 mg. of vitamin without toxin did not show indications of scurvy until about 10 days after the last injection, distinct differences in toxin resistance may be considered to have been manifested previous to the appearance of scurvy. The contrast between the high and low nutritive levels receiving toxin was clearly evident (compare curve B with B<sup>1</sup>, and curve C with C<sup>1</sup>) before the 0.3-mg. group of controls had shown any significant weight deviation from the 1.5-mg. group of controls (compare curves A and A<sup>1</sup>). It will be seen that the two 0.3-mg. groups receiving toxin (curves B<sup>1</sup> and C<sup>1</sup>) were losing weight rapidly 13 days after the first injection, although the 0.3-mg. control group without toxin (curve A<sup>1</sup>) did not begin to show a loss in weight until 19 days after the first injection. The contrasts in local inflammatory reactions were more marked than the differences in body weight.

The measurement of oxygen consumption by liver and kidney tissue is recorded in table 2. The animals used for this purpose were taken at the end of the experimental period shown in figure 1, i.e., during the third week after the last injection. The controls on the lower nutritive level (curve A<sup>1</sup>) and those on curve B showed only a slight depression below the values for the normal group (curve A). The animals from curves B<sup>1</sup> and C exhibited a reduction of approximately 15 per cent in oxygen uptake for kidney tissue. Apparatus was available for only two to four animals from each

group. The animals from group C<sup>1</sup> died before manometric measurements were started.

To check the results of the previous experiments and to eliminate the possible interference of factors other than vitamin C in orange juice, a further series (summarized in fig. 2) was run, in which the pure vitamin was fed in aqueous

TABLE 2

*Oxygen consumption in cubic millimeters per 100 mg. of wet tissue at end of 2 hours at 37°*

NUMBER OF ANIMALS	VITAMIN LEVEL	AMOUNT OF TOXIN INJECTED	CORRESPONDING CURVE IN FIGURE 1	LIVER	KIDNEY
	<i>mg.</i>				
2	1.5	0	A	90.3	201.9
4	1.5	4 doses each of 0.3 M.L.D.	B	91.7	193.6
2	1.5	4 doses each of 0.5 M.L.D.	C	75.2	175.5
3	0.3	0	A <sup>1</sup>	87.5	189.7
3	0.3	4 doses each of 0.3 M.L.D.	B <sup>1</sup>	88.0	170.4

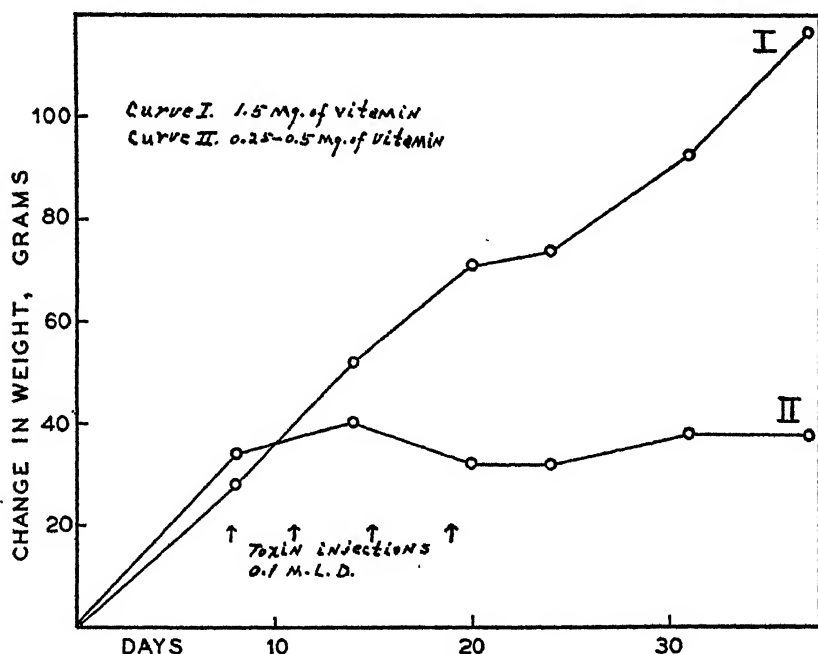


Fig. 2 Effect of vitamin C feeding level upon the resistance of guinea pigs against small dosages of diphtheria toxin.

solution. One group of eight animals (curve I) received 1.5 mg. of vitamin daily. The other group of nine animals (curve II) was depleted for 8 days and then given 0.25 mg. of vitamin C per day for 2 weeks, after which the vitamin intake was increased to 0.50 mg. per day to avoid the onset of scurvy. Each animal of both groups was injected subcutaneously with 0.1 M.L.D. of diphtheria toxin on the fourteenth, seventeenth, twenty-first and twenty-fifth days. The minimum, average and maximum weights of animals represented by curve I at the time of the first injection were 350, 399 and 430 gm. respectively, while the corresponding values for curve II were 325, 377 and 415 gm. Those in curve I (final average weight 464 gm.) showed very little deviation from a normal growth rate, and at the end of the test period showed practically complete healing at the sites of injection. Those in curve II (final average weight 375 gm.) were unable to resume growth when placed upon the 0.5-mg. level, and local congestion was evident as a result of the injections. The contrast in degree of inflammatory reaction and rate of healing was as marked as when orange juice was used as a source of the vitamin.

#### SUMMARY

Guinea pigs under controlled conditions and receiving definite daily quantities of vitamin C at abundant, protective, and sub-protective levels, were given subcutaneous injections of standardized diphtheria toxin in 0.1, 0.3, 0.5 and 1.0 M.L.D. When the animals were partially depleted of their vitamin C reserves without showing external signs of scurvy, the survival time was shortened about 50 per cent and the loss in body weight was more severe. Hemorrhage and necrosis at the site of toxin injections were more marked in the latent scurvy condition. The decrease in oxygen consumption capacity of liver and kidney tissue after toxin injection was in the range of 5 to 15 per cent. It is evident from the study that there is a wide zone of vitamin C deficiency, without the appearance of scurvy, where physiological processes are subnormal and the animal is more sensitive to injury from bacterial toxin.

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# THE INFLUENCE OF VITAMIN C LEVEL UPON RESISTANCE TO DIPHTHERIA TOXIN

## II. PRODUCTION OF DIFFUSE HYPERPLASTIC ARTERIOSCLEROSIS AND DEGENERATION IN VARIOUS ORGANS

MAUD L. MENTEN AND C. G. KING

*Departments of Pathology and Chemistry, University of Pittsburgh, Pittsburgh*

ONE PLATE (FOUR FIGURES)

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Repeated attempts to produce in experimental animals a diffuse hyperplastic arteriosclerosis similar to that encountered in human pathology has thus far not met with signal success. In the course of microscopic examination of tissue removed from guinea pigs maintained on diets deficient in vitamin C and treated with diphtheria toxin, as described in the previous paper, hyperplastic vascular lesions were constantly obtained. The microscopic description of these lesions forms the basis of the present communication.

Lack or absence of vitamin C gives rise to definite degenerative changes in the highly specialized organs as well as developmental irregularities in bones and teeth. Among the former may be cited granular degeneration with hemorrhage in the adrenal cortex, fatty degeneration and hemorrhage in the cardiac musculature, central toxic necrosis of liver, atrophy of germinal epithelium of the testis and ovary, ulcers of the intestinal mucosa, and degeneration, hemorrhage and atrophy throughout the entire skeletal musculature. These have been described by several investigators (Bessey, Menten and King, '34; Meyer and McCormick, '28; Lindsay and Medes, '26). The addition of bacterial toxins to the circulation hastens and intensifies the development of such processes

but it is not our purpose to give a detailed amplification of such degeneration. It is desired rather to describe an additional phenomenon, which results when the guinea pig, an animal incapable of synthesizing vitamin C, is maintained on an inadequate vitamin C diet, and coincidentally subjected to the continuous action of diphtheria toxin. A measure of the general effect of such a regime has already been indicated in the loss of weight and decreased duration of life reported in the previous paper.

#### TECHNIC AND RESULTS

The material obtained was removed at necropsy from the various animals, whose treatment has been outlined in the previous paper. Tissues fixed immediately after death in Zenker's solution and 10 per cent formalin were routinely stained with hematoxylin and eosin. This stain was supplemented by Sudan iv and Verhoeff's and Weigert's elastic tissue stain. Although more or less degenerative change occurred throughout the body, only lesions in lung, heart, liver, spleen, kidney, pancreas and brain will be described. Hyperplastic arteriosclerosis was observed in all of these except the heart and brain.

The lungs were fairly normal except for degeneration in the bronchial wall, many thickened vessels and frequently irregularly distributed areas of collapsed lung tissue, whose blood supply had been materially reduced by arteriosclerotic vessels. Sclerotic vessels were the outstanding pathologic feature of microscopic sections of lung. Indeed, this vascular lesion was better demonstrated in lung than in any other organ. The enormously thickened vessels, the walls of which had impinged upon and decreased the lumina to a minimum, contrasted strikingly with the surrounding loose alveolar pulmonary tissue (figs. 1 and 2). The thickening was most marked in the medium-sized arterioles but was also observed in larger arteries. The thickening was due to a diffuse hyperplasia of the connective tissue, mainly of the media. It was fairly uniform and continuous except at the point of origin of

the smaller branches. Occasionally the hyperplasia did not include the entire circumference but was lacking on that side of the vessel which was buttressed by the contiguous bronchiole. The internal elastic lamina was swollen, but retained a considerable affinity for the elastic tissue reagents. In some of the smaller arteries the widespread degeneration of the elastic tissue had rendered the identification of the internal elastic lamina impossible and when such vessels underwent hyperplasia it was difficult to apportion the amount belonging to media or intima. In many such vessels the degeneration throughout had been so rapid and extensive that compensatory hyperplasia had not taken place. Although the retrograde change in the elastic tissue was the conspicuous feature, the earliest change was in the smooth muscle. Of all the medial elements the muscle cells appeared to be the most susceptible to the toxin and it was in them that the pathologic process was initiated.

The first detectable microscopic change was a cloudy swelling gradually progressing to a necrosis with pyknosis and loss of staining of the smooth muscle cell. With large doses of toxin the disintegration of the muscle cells was often so rapid that no trace of the process was discernible. Sometimes a few remnants of pyknotic nuclei remained. The next step in the retrograde process was the involvement of the elastic tissue. These fibers began to take on a granular appearance with loss of their distinct marginal outline. Variations in the degree of staining with Verhoeff's and Weigert's elastic tissue stains began to appear. The fibers lost their wavy contour and tended to lie in straight rows. Some of the fibers did not stain at all. Finally, clumping and disintegration took place and many of the elastic fibers disappeared completely. The process was one of gradual deterioration. Splitting and fraying of the elastic tissue were not observed. These changes were not accompanied by an appreciable fat phanerosis. Furthermore, calcification did not occur.

The early disappearance of smooth muscle associated with loss of resiliency and tone in the elastic fibers induced a compensatory hyperplasia of the connective tissue of the arteries.



In the larger vessels the retrograde changes began in the central zone of the media, and then advanced inward and outward. When the entire media became involved there was often a thinning of the vessel wall, due to compression of the residual elastic tissue fibers so that they were pressed and clumped together. Often between these could be seen necrobiotic granular tissue which stained irregularly. This was constantly seen in the wall of the aorta and pulmonary vein. Compensatory fibrosis does not occur in these vessels, but an attempt to counteract this loss of supporting tissue and reduction in resiliency was seen in the formation of small nodules of cartilage. These were accessory to the aortic nodules found in older pigs and were usually situated at a greater distance from the roots of these vessels. Metaplasia of connective tissue into cartilage could be seen in various stages. Although the vascular lesions are of the productive instead of the degenerative type produced by Klotz ('06), Bailey ('17), and Duff ('32) with diphtheria toxin in rabbits, they possess certain features described by these authors. The degenerations taking place in smooth muscle and elastic tissue are common to both. The intimal changes described by them in the aorta were not seen. The small size of the guinea pig's aorta may have made these inconspicuous, and hence overlooked. The same etiologic factors preceding arterial thickening, namely, loss of smooth muscle and degeneration of the elastic fibers are responsible for the thinning of the bronchial wall with subsequent crumpling, buckling and infolding, occasionally seen in sections. Compensatory fibrosis was lacking and the degenerations in these structures were often accompanied by retrograde changes in the contiguous lymphocytic nodules which stain very intensely.

Myocardial degeneration resulted in widespread loss of heart tissue which manifested itself in the grossly shrunken and wrinkled visceral pericardium. The progressive stages beginning with loss of cross striation and ending with complete dissolution of the muscle cell could be followed in various parts of the heart. Many small collapsed arterioles remained as closely approximated lines of pyknotic nuclei.

Their closure added to the rapid dissolution of areas of myocardial substance. The visceral pericardium occasionally showed slight degrees of fibrosis or fatty infiltration. The valves did not show significant changes. In some cases there was a slight medial thickening of the larger coronary vessels near the base of the heart which was probably unrelated to the experimental procedure. This was the only evidence of hyperplastic arteriosclerotic change in the myocardium observed in any of the animals. This lack of sclerosis and the presence of collapsed degenerated capillaries in the heart, is in sharp contrast to the marked vascular thickening occurring in the lungs.

In the liver the arterial thickening introduced an additive factor in the progressive cellular degeneration and lobular necrosis obtaining in scorbutic and sub-scorbutic conditions. The hyperplasia of the radicles of the hepatic artery never attained the extent seen in the pulmonary vessels. This can probably be partly explained by the resistance offered by the surrounding dense liver tissue. Also, only comparatively few of the interlobular vessels were thickened. The walls of the thickened interlobular arteries were fairly uniform in thickness and had a hyaline appearance. Their lumina were frequently so reduced that they were almost occluded (figs. 3 and 4).

In the spleen the arteriosclerosis was best seen in those branches of the splenic artery encircled by the lymphatic sheaths, especially in the center of the malpighian corpuscles. The central vessels of many of these structures, however, showed no hyperplasia and the distribution of arteriosclerosis was irregular. The appearance of the thickened vessels was similar in size and appearance to those seen in the liver.

The distribution of the sclerotic vessels in the kidney was irregular. It was difficult to determine with certainty in which of the smaller divisions of the renal artery the hyperplasia had taken place. The interlobular arteries and arteriolae rectae were apparently constantly involved. In a few instances the widespread cellular degeneration result-

ing in reduction of the number of secreting units, with compensatory dilatation of the remaining tubules suggested that the entire arterial tree had been involved. The destructive quality of the toxin per se in producing similar retrograde process in renal parenchymatous tissue should be kept in mind in evaluating the consequences of vascular occlusion. In only one animal were morphologic changes in the glomeruli observed which approximated those seen in the human arteriosclerotic kidney. The occluded glomeruli in this case, however, appeared hyaline resembling some of the sclerotic hepatic vessels.

In those pigs receiving small repeated injections of diphtheria toxin productive vascular lesions were not found. Microscopic examination was made on tissue from six pigs maintained on 0.25 mg. vitamin C daily and given 0.1 M.L.D. diphtheria toxin on the fourteenth, seventeenth, twenty-first and twenty-fifth day after the beginning of the deficient diet (see fig. 2 previous paper). Two of the pigs, killed 1 day after the fourth injection, showed a moderate thickening in some of the larger pulmonary arteries. The vessels of the remaining four pigs, killed 16 days after the last injection, appeared normal. From these observations it is inferred that such dosage is not sufficient to produce vascular sclerosis. It is possible that small repeated injections made at 3- or 4-day intervals may induce a degree of immunity against the diphtheria toxin which modifies its toxic effect.

Evidence of arteriosclerosis in the pancreas was observed in a few medium-sized vessels. The picture here resembled that described in pulmonary vessels of similar size. The readiness with which vascular thickening can be produced with diphtheria toxin in guinea pigs and especially in vitamin C-deficient pigs would suggest that some evidence of hyperplastic arteriosclerosis might be found in normal pigs which are fairly susceptible to spontaneous infections. Medial fibrosis of a slight degree was observed in the large pulmonary vessels of some of the control animals. This was more conspicuous in two or three of the pigs which had been

maintained on a scorbutic diet and we believe can be attributed to a concurrent infection. In no instance have we observed spontaneous vascular thickening in organs other than the lungs. Of the two main factors, namely, vitamin C deficiency and diphtheria toxin, concerned in the production of the hyperplastic arteriosclerosis, the influence of the toxin appears to be the easier to evaluate from microscopic evidence. As has already been indicated, the four 0.1 M.L.D. of diphtheria toxin injected at 4-day intervals into pigs maintained on a daily vitamin ration sufficiently high to prevent obvious scorbutic symptoms (0.25 to 0.50 mg. daily) was not sufficient to produce a vascular thickening even in the more responsive pulmonary arteries. With the next higher diphtheria dosage used (0.3 M.L.D.) diffuse hyperplastic arteriosclerosis was produced regardless of the vitamin C level. About 10 days are required for the development of the sclerosis. That lack of, or decrease in, vitamin C is one of the indispensable factors in the pathogenesis of this lesion seems assured from the ease with which the lesion is produced in the guinea pig, an animal incapable of synthesizing this vitamin, in contradistinction to the practical impossibility of eliciting it in other experimental animals which can synthesize vitamin C. The smooth muscle content of even large vessels of the muscular type is small in amount and difficult to separate from the other cellular constituents. It is impossible to obtain an amount sufficient for titration of vitamin C from guinea pig vessels. In an effort to ascertain the vitamin C content of smooth muscle, vitamin C titrations were made on tissue from uterus and myomata removed at operation and on post-mortem bladder, uterus, and large and small intestinal wall freed as far as possible from mucosa and peritoneal covering.

Titration on three human uteri and two myomata gave vitamin C values ranging between 0.1 and 0.2 mg. per gram. The intestinal values from two autopsy cases dying from infection were 0.02 and 0.07 with the higher figure for the large intestine. Determinations on bladder and uterus in the

last two cases were in the same range, about 0.03 mg. per gram. Slightly higher values were obtained from intestinal muscle of the guinea pig. The lower figures are probably explainable on vitamin depletion due to infection. In any case it would appear reasonably certain from even these few observations that the vitamin C content of normal smooth muscle is comparatively low and that it can be readily reduced in amount.

Besides the productive lesions already described, degenerative lesions in the brain and pancreas are worthy of note. The large ganglion cells of the cerebral hemispheres and cerebellum were swollen and showed varying degrees of chromatolysis, and there was frequently an associated edema.

The characteristic lesion in the pancreas was hydropic degeneration of the islets of Langerhans. Similar lesions were first produced in the guinea pig by Thomas ('24) working with *B. enteritidis* in our laboratories. Under low power magnification the transparency and pale color of the islets were in striking contrast to the surrounding deeply stained acinar units. On high power examination the reason for the pale color was obvious. The beta cells were enormously swollen and their cytoplasm was packed with large watery vacuoles which were outlined by the meshes of a faintly staining network. The nuclei were swollen and vesicular, although rupture of such cells was rarely seen. While the majority of the beta cells showed this hydropic degeneration, a fair number were shrunken and had a finely granular cytoplasm and a pyknotic nucleus. These two types of cellular degeneration were seen in all pigs which had received single or repeated doses of 0.3 M.L.D. or higher dosage of toxin, if the animal were killed or died during the acute intoxication. The alpha cells appeared fairly normal. Up to a certain point the hydropic degeneration was reversible because in animals which had recovered, the islets appeared normal. In pigs receiving four injections of 0.1 M.L.D. of toxin at 4-day intervals, typical hydropic degeneration was not seen, even in the animals killed shortly after the fourth dose. In

a few instances, intensity differences in staining, developing with hematoxylin and eosin, sufficed for the differentiation of the alpha and beta cells.

Various reports (Homans, '14; Allen, '22; Menten and Manning, '25; Menten, '26) have indicated a correlation between the experimentally produced hydropic degeneration in the beta cells and hyperglycemia of experimental animals. With this in mind, a series of sugar tolerance tests were made for several weeks following the 0.3 and 0.5 M.L.D. injections of toxin. The animals, after having been deprived of food for 5 to 12 hours, were given 0.5 to 1 gm. of glucose orally. Blood samples of 0.1 cc. were taken from the marginal ear vein and the reducing sugar content determined by Folin's micro method. A lowering of sugar tolerance was found with values in the range of 250 to 400 mg. per 100 cc. of blood, persisting for 2 to 3 hours. With increasing intoxication and parenchymatous damage the further lowering of the sugar tolerance became marked. With the disappearance of the symptoms and restitution of the pig to normal physiologic conditions following recovery from the toxin, there was a return to normal tolerance.

It is interesting that approximately the same amount of diphtheria toxin is required for the production of the arteriosclerosis as for the lesions in the islets of Langerhans.

#### DISCUSSION

A comparison of the vascular lesions produced in rabbits and guinea pigs by injections of diphtheria toxin reveals fundamental similarities in the early stages of the pathogenesis. Thus the degenerative changes occurring in the medial smooth muscle and elastic tissue of the rabbit, as described by Klotz ('06), Bailey ('17), and Duff ('32) can be duplicated in the guinea pig. The conspicuous difference is in the connective tissue response in either animal. This morphologic difference is paralleled by the difference in vitamin C metabolism in the two animals. While the relationship of vitamin C to connective tissue is complex, the investigations of Wolbach and Howe ('26) demonstrate that in the

guinea pig an adequate vitamin C intake is indispensable for the formation and integrity of collagen. Presumably respiratory tissue metabolism is intimately adjusted to such a connective tissue relationship and herein lies the basis for the unique vascular response in the guinea pig.

Apparently variation in vitamin C metabolism is a potent factor in determining the degree of connective tissue hyperplasia in the affected arteries. Another factor concerned in this hyperplasia is the anatomic structure of the vessel, particularly the histologic constitution of the media. Degrees of degenerative change in the medial elements, together with volume and force of blood flow in the arterial bed, are further modifying factors.

A number of preliminary experiments have demonstrated that the infectious agent may vary widely since the substitution of pneumococcus, staphylococcus, *B. coli*, *B. typhosus*, *B. enteritidis* and green and hemolytic streptococci for diphtheria toxin yields comparable results.

It is noteworthy that the guinea pig is one of the few animals which shares with man the latter's inability to synthesize vitamin C. The production of hyperplastic arterial thickening in the guinea pig by a combination of dietary vitamin C deficiency and infection it is believed is not without significance in relation to the origin of diffuse hyperplastic arteriosclerosis in the human, despite the fact that morphologic differences exist between the two lesions. The intimal thickening characteristic of human arteriosclerosis is lacking and the selective organic distribution is not identical in the experimental form. Subsequent investigation will possibly reveal the reason for these differences. It also remains to be determined in detail how the two components, namely, vitamin C deficiency and the infectious agent, which produce their cumulative effect on the vessel wall, exert their individual influence.

Another feature brought out by our experiments which would seem to have a bearing on human pathology is the association of degeneration in the islets of Langerhans and

lowered sugar tolerance with the arteriosclerosis. The high incidence of diabetes with human arteriosclerosis has long been a subject of clinical speculation. Recently Yannet, Darrow, Goldfarb and Cary ('33, '34) have reported disturbances in blood sugar following injections of diphtheria toxin into rabbits and have interpreted the subsequent hyperglycemia as due chiefly to liver injury with resultant failure of liver balance. The fact that severe parenchymatous damage in the liver with insignificant pathology in the pancreas is sufficient to give rise to hyperglycemia in rabbits has been attested by many workers. Following injection of the *B. enteritidis*, its toxic products and peptone, we (Menten and Manning, '27; Menten and King, '30) have obtained such a phenomenon. Zeckwer ('25) and others (Delafield, '31, '32, '34) have shown that injection of various members of the colityphi group will produce a similar picture. Rabbits, however, are not subject to vitamin C deficiency and their tissue injury would not be complicated by disturbances arising from this source. Much of the effect on sugar tolerance observed by us may be due to liver and kidney injury but the changes produced in the beta cells of the islets of Langerhans are indicative of an additional injury to the insulin elaborating mechanism. We believe that the ease with which islet cell lesions are produced in the guinea pig is significant. The complexity of functional relationships between liver and pancreas make it necessary to continue the experiments through a longer time period before arriving at a definite conclusion. This work is being continued in order to determine whether a permanent islet damage associated with hyperglycemia and glycosuria can be produced.



## SUMMARY

Guinea pigs maintained on diets deficient in vitamin C, and injected with sublethal amounts of diphtheria toxin showed diffuse hyperplastic arteriosclerosis in lungs, liver, spleen and kidneys. No vascular thickening was observed in the heart. Such animals also developed hydropic degeneration of the islets of Langerhans and an associated hyperglycemia and lowered glucose tolerance. With recovery of the animal the pancreatic lesions disappeared. The significance of these findings is discussed.

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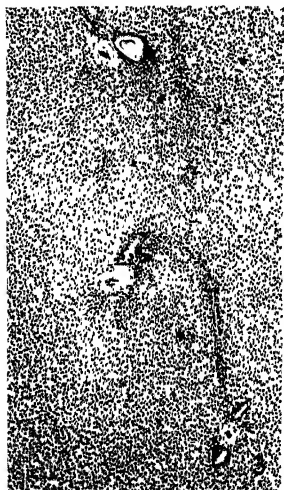
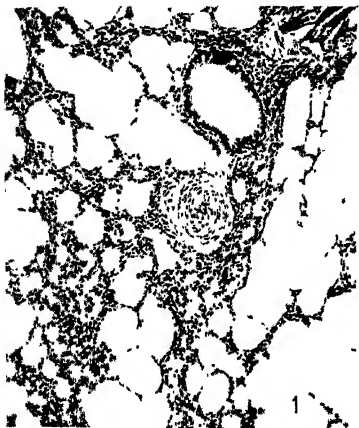
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## PLATE 1

### EXPLANATION OF FIGURES

Guinea pig (initial weight 345 gm.) fed daily on 0.3 mg. vitamin C, as orange juice, and given four injections of diphtheria toxin, each containing 0.3 M.L.D. on the sixteenth, twenty-fourth, twenty-eighth and thirtieth days. Animals died on thirtieth day.

- 1 Arteriosclerotic vessel of lung.  $\times 100$ .
- 2 Arteriosclerotic vessel of lung.  $\times 250$ .
- 3 Arteriosclerotic vessel of liver.  $\times 60$ .
- 4 Arteriosclerotic vessel of liver.  $\times 500$ .





# ASCORBIC ACID (VITAMIN C) IN SPROUTED OATS <sup>1</sup>

RALPH BOGART AND J. S. HUGHES

*Kansas Agricultural Experiment Station, Manhattan*

TWO FIGURES

(Received for publication April 20, 1935)

During the winter months when other green feed is not available, sprouted oats has proved a satisfactory source of vitamin C for guinea pigs. After trying many ways of sprouting oats for a colony<sup>2</sup> of about 1000 animals, the following method has been in use the past few years. After soaking the oats in water for 5 hours, they are placed in large shallow pans, where they are well stirred and watered twice a day for 7 days. At this stage the shoots have made such development (fig. 1) that stirring is no longer practical. To continue the growth without stirring results in matting of the roots which renders the oats less desirable for feeding. Also, after the seventh day, increasing difficulty in preventing mold and bacterial development is met. For these reasons it has been found desirable to feed the oats at that stage of growth. They are fed at the rate of about 40 gm. for each animal, which amounts to approximately five hundred seedlings. Results obtained with this large colony of guinea pigs show that this method of feeding sprouted oats provides the animals' requirement of vitamin C.

Since a convenient chemical method (Bessey and King, '33) for quantitative analysis of vitamin C is known, it was decided to determine the amount of vitamin C that was actually

<sup>1</sup> Contribution no. 112 from the Department of Animal Husbandry. Contribution no. 189 from the Department of Chemistry.

<sup>2</sup> This colony is maintained by the Department of Animal Husbandry for genetic studies.

being fed. In addition, the vitamin C content of oats was determined at the different stages of sprouting from the first to the thirteenth day. Oats used for these determinations were sprouted in the same manner as was used in sprouting the oats for feeding the guinea pigs, except some of the pans were allowed to develop for 13 days. One group of pans was kept in the dark after the seventh day.



Fig. 1 Showing development of oat seedlings on the seventh day of sprouting.

The ascorbic acid was determined by extracting a 5-gm. sample with 25 ml. of 3 per cent trichloroacetic acid, centrifuging and titrating an aliquot of the clear filtrate with standardized 2-6-dichlorophenolindophenol.

The results which are graphically shown in figure 2 indicate a steady increase in vitamin C content up to the tenth day. After this time there seems to be a slight decline in the vitamin C content of the material under the conditions of this ex-

periment. There was very little difference in vitamin C content of the oats sprouted in the light and those in the dark, although there was considerable difference in pigmentation of the sprouts.

The vitamin C content of the various parts of the plant was determined from a sample at the seventh day. These analyses showed that over 90 per cent of the vitamin C was

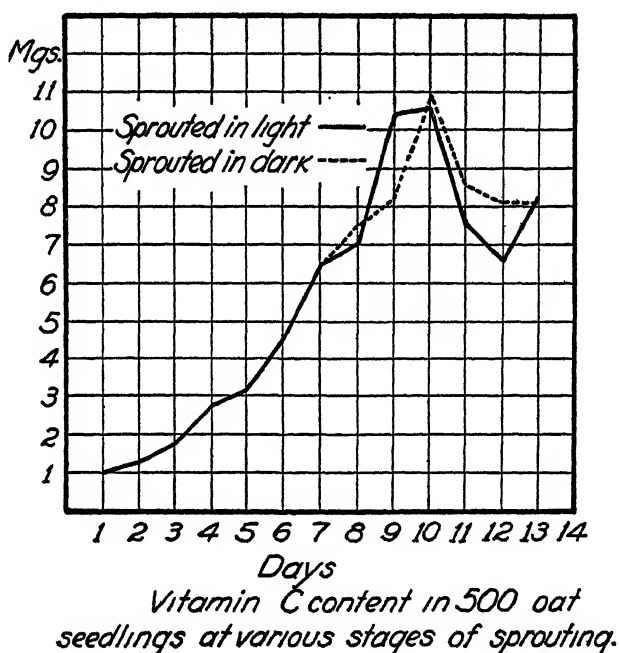


Figure 2

contained in the epicotyl. There was very little difference between the vitamin C content of the root and that of the kernel at this stage of development. If any difference, there was slightly more in the kernel.

These results indicate that each guinea pig in this colony obtains between 6 and 7 mg. of vitamin C from 40 gm. of sprouted oats a day. This would indicate that the amount of sprouted oats might be considerably reduced without endangering the animals from a deficiency of vitamin C.



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# STUDIES OF CRYSTALLINE VITAMIN B

## VI. THE EFFECT OF GRADUATED DOSES ON PIGEONS

ROBERT E. WATERMAN AND MARION AMMERMAN

*Department of Physiological Chemistry, Teachers College, Columbia University*

### THREE FIGURES

(Received for publication December 29, 1934)

The rat has been the test animal for the past few years in this laboratory in the development of a suitable procedure for the isolation of vitamin B(B<sub>1</sub>) and as a means of assay of the crystalline material. In view of the wide use of birds as B test animals in other laboratories, it was considered advisable to investigate the curative effect of the vitamin on pigeons.

Adult birds were put on a diet of autoclaved whole wheat ad lib. plus oyster shell. The wheat had been autoclaved for 4 hours at 15 pounds' steam pressure. Upon the onset of polyneuritis, usually after 3 to 4 weeks, when the birds had declined 20 to 25 per cent in weight, dilute ethyl alcohol solutions of crystalline vitamin B hydrochloride were administered orally by pipette.

Doses as low as 3  $\gamma$  have always brought about some improvement and in some cases have effected cures. Four  $\gamma$  is generally curative and 5  $\gamma$  has never failed to cure a bird with polyneuritis. Pigeons respond to a curative dose more quickly than do rats; a bird which is prostrate and shows violent head retraction is often able to stand within 2 hours and seems completely cured on the following day. The pigeons used have not been standardized as to response (Kinnersley, Peters and Reader, '28) and only a limited number have been used, which makes the data on the effect of magnitude of dose on the length of cure unsatisfactory.

Rats show a remarkable response to high levels of dosage with crystalline vitamin B (Waterman and Ammerman, '35). For comparative purposes the effect of a range of doses of the crystals on the weights of adult pigeons which had been depleted on a diet of autoclaved wheat has also been tested. The birds were fed whole wheat ad lib. plus oyster shell over a period of 2 to 3 weeks in order to set a normal weight, which ranged from 300 to 350 gm. Autoclaved wheat was then substituted for the whole wheat and after 21 days, when they had declined about 25 per cent in weight, the vitamin addendum was started. To one group 5  $\gamma$  was given daily until the initial weight increase occasioned by the administration of the vitamin had given way to a further decline. The dosage was increased to 20  $\gamma$  daily and kept at that amount until the birds' weights were again level. Up to this point very little weight gain had been effected, the birds being but slightly heavier than when the 5  $\gamma$  dosage was started. Eighty  $\gamma$  daily was then fed with some further gain, but the weights leveled out at about 80 to 90 per cent of normal and additional increase to 160  $\gamma$  daily was not adequate to bring the birds back to their former weight. Another depleted group was started at 10  $\gamma$  daily and raised to 40  $\gamma$  daily with similar results. The vitamin addendum was then stopped and after a short period when the weights had again turned down whole raw wheat was substituted for the autoclaved wheat.

Figure 1 gives the weight curves of individual birds typical of each of the two groups of four each and figure 2 shows graphically the averages of results which have been obtained. At the 5  $\gamma$  dosage the pigeons were free from polyneuritic symptoms, but were unable to hold their temporary weight gains. With increasing amounts of B up to about 80  $\gamma$  per day, the average weight level is successively raised. The behavior of the birds varied rather widely from individual to individual, but none was able to regain its normal weight even with the maximum amount of vitamin administered, 160  $\gamma$  daily. However, in every case the change in diet to raw whole wheat always led to an immediate steady weight increase which restored the birds quickly to their normal weight.

These results constitute further evidence of the existence in wheat of a factor corresponding to that postulated by Williams and Waterman ('28) under the designation  $B_3$ . In

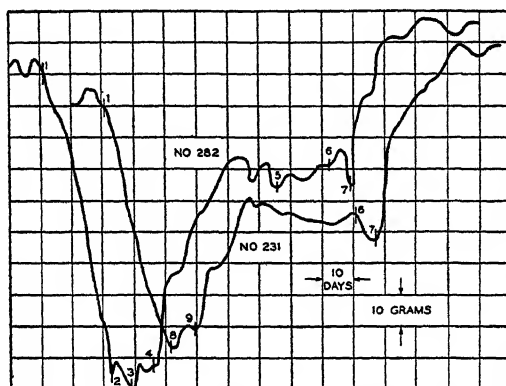


Fig.1 Weight curves of typical pigeons on increasing vitamin B dosage. At point 1 autoclaved wheat was substituted for raw wheat. At point 2, 5  $\gamma$  of the crystalline vitamin was fed daily, at point 3 the dosage was increased to 20  $\gamma$ , at point 4 to 80  $\gamma$  and at point 5 to 160  $\gamma$ . At point 8, 10  $\gamma$  was fed daily and at point 9 the dosage was raised to 40  $\gamma$ . At point 6 the vitamin dosage was stopped and at point 7 the birds were again put on a diet of raw wheat.

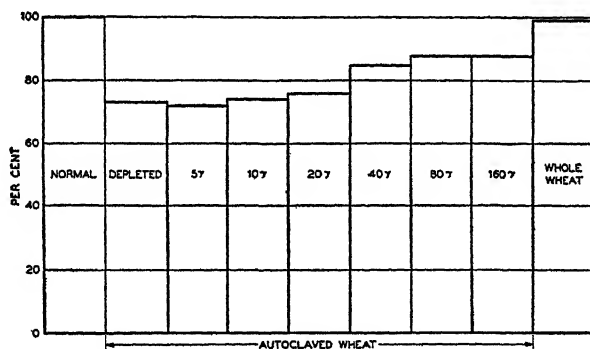


Fig.2 Average weight level of pigeons on various dosages of crystalline vitamin B hydrochloride.

a further effort to throw light on the existence or non-existence of the so-called  $B_3$ , feeding tests on depleted pigeons were made with increasing amounts of raw whole wheat as addenda. Five birds at full weight were given autoclaved

wheat for 3 weeks, after which time they were about 20 per cent below top weight. The autoclaved wheat was continued ad lib. and a daily supplement of 5 gm. of whole raw wheat was added. In each of the birds under test a prompt but rather small weight increase was effected. The birds soon reached weight equilibrium and the addendum of whole wheat was increased to 7.5 gm. per day which produced a similar weight rise and leveling. The addendum was increased to 10 gm. daily which was sufficient to bring two of the birds back

TABLE 1

*Weight response of depleted pigeons to supplements of raw wheat*

	NUMBER				
	22	23	38	20	28
Weights in grams on:					
Raw wheat ad lib.	330	350	325	345	340
Autoclaved wheat ad lib. for 21 days.	287	274	243	268	278
Autoclaved wheat ad lib. + 5 gm. raw wheat	304	298	266	235	284
Autoclaved wheat ad lib. 7.5 gm. raw wheat	317	324	294	314	308
Autoclaved wheat ad lib. 10.0 gm. raw wheat	326	351	313	321	326
Autoclaved wheat ad lib. 12.5 gm. raw wheat	...	...	325	324	333
Raw wheat ad lib.	...	...	...	324	333

to normal weight. An increase to 12.5 gm. of whole wheat daily restored the weight of a third bird, while the remaining two did not attain their previous top weights<sup>1</sup> even when the whole wheat was given ad lib. The results are given in table 1 and typical weight curves are given in figure 3.

From our own as well as other data (Chase and Sherman, '31), one would judge the B content (calculated as the hydrochloride) of whole wheat to be approximately 5  $\gamma$  per gram. Assuming this to be true, 10 gm. of whole wheat daily would be equal to 50  $\gamma$  of B. When the B is administered in the form of whole wheat, 50 to 62.5  $\gamma$  daily is necessary to restore

<sup>1</sup> A later survey of our results indicates that these top weights were 'super-normal' and due to previous liberal dosage with crystalline B<sub>1</sub> followed by a whole wheat diet. The hold-over effect on weight of large amounts of B<sub>1</sub> has been noted consistently.

the birds to normal weight levels. When the B is fed as a crystalline chemical compound even 160  $\gamma$  per day is insufficient for complete weight restoration. The results reported furnish additional evidence that there is a B complex factor other than B(B<sub>1</sub>) needed for the complete nutrition of pigeons. However, regardless of the effects of B<sub>3</sub>, large amounts of pure vitamin B lead to higher weight levels in adult pigeons as they do in rats.

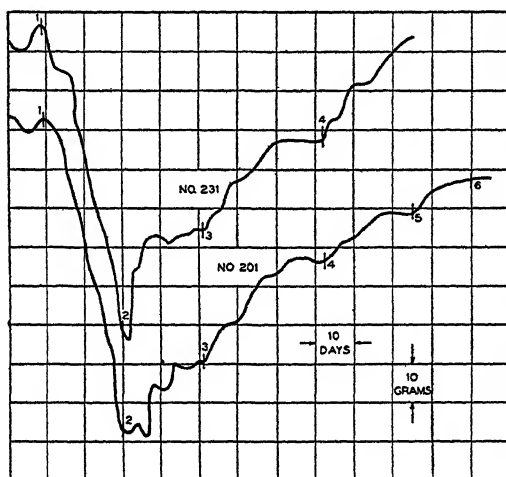


Fig. 3 Weight response of typical depleted pigeons to supplements of raw wheat. At point 1 autoclaved wheat was substituted for raw wheat. At point 2, 5 gm. of raw wheat were fed daily. At point 3 the dosage of raw wheat was increased to 7.5 gm., at point 4, to 10 gm. and at point 5 to 12.5. At point 6, whole wheat was fed ad lib.

#### SUMMARY

1. The minimum dose of vitamin B hydrochloride which cures polyneuritis in pigeons is approximately 4  $\gamma$ .

2. Increasing amounts of B lead to higher weight levels in previously depleted pigeons up to 80  $\gamma$  per day, but even 160  $\gamma$  is inadequate to bring the birds back to normal weight.

3. The significance of the results in relation to 'vitamin B<sub>3</sub>' is discussed.

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# THE RELATIONSHIP OF THE VITAMIN D INTAKE OF THE HEN TO THE ANTIRACHITIC POTENCY OF THE EGGS PRODUCED<sup>1, 2</sup>

N. B. GUERRANT, ELIZABETH KOHLER, J. E. HUNTER AND  
R. R. MURPHY

*Departments of Agricultural and Biological Chemistry and Poultry Husbandry,  
Pennsylvania State College*

## ONE FIGURE

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Hess ('23) found egg yolk to be one of the few important natural sources of vitamin D. Later, Hughes, Payne, Titus, and Moore ('25) conducted an investigation in which they fed egg yolks of known source to young chicks. These authors came to the conclusion that the vitamin D content of the egg depends on the diet of the hen, as well as on the light to which she is exposed. Bethke, Kennard, and Sassaman ('27) found that eggs from range hens which received direct sunlight were ten times as potent, antirachitically, as eggs produced by hens receiving the same basal diet, but which were confined indoors where they received only light that had been filtered through glass. These authors also found that 2 per cent of cod liver oil, when added to the basal diet, induced storage of five times as much vitamin D in the egg as did the basal diet alone or the basal diet supplemented by alfalfa ad libitum.

The investigations of DeVaney, Munsell, and Titus ('33) are the most recent reported in the available literature on this subject. These investigators made biological assays on

<sup>1</sup> We wish to express our indebtedness to the National Oil Products Company, Harrison, New Jersey, for financial assistance in carrying out this investigation.

<sup>2</sup> Publication authorized by the director of the Pennsylvania Agricultural Experiment Station April 16, 1935, as technical paper no. 686.



egg yolks produced under controlled conditions, using young rats as the experimental animals. The technic employed is quite comparable to that in use at the present time. These investigators concluded that vitamin D from cod liver oil is more efficiently stored in the egg yolk than is vitamin D from an equivalent amount of viosterol, and that the vitamin D potency of egg yolk can be increased within certain limits by increasing the vitamin D intake of the hen.

Unfortunately, the data obtained in the investigations reported above cannot be compared on the basis of actual vitamin D unitage, owing to variations in the biological technic employed. It seemed desirable, therefore, that further efforts should be made toward securing additional information concerning the antirachitic potency of eggs produced under carefully controlled conditions. Since an investigation was already in progress at this institution which offered the opportunity of obtaining the eggs for such a study, it was highly desirable that an investigation be carried out at this time.

With the above facts in mind, the studies herein reported were initiated to determine the antirachitic potency of eggs produced by groups of hens which received diets varying only in their antirachitic potency.

#### EXPERIMENTAL

The eggs used in these studies were obtained from groups of hens which were being used in a series of nutritional studies in the department of poultry husbandry. These birds were denied access to sunlight and were maintained on a rachitogenic basal all-mash ration which was supplemented by varying amounts of vitamin D in the form of fortified cod liver oil, viz. (8D).<sup>3</sup> Further information concerning the above investigation has been published elsewhere (Murphy, Hunter, and Knandel, '34).

The collection and the sampling of the eggs, and the preparation of the egg yolk samples were carried out in the following manner: The eggs were collected daily, weighed, labeled,

<sup>3</sup>This cod liver oil contained 100 Steenbock vitamin D units per gram, or approximately 8D potency.

and stored in a cool dark place until the end of the collection period. A complete record was kept for each bird in each of the several groups, in order that only representative eggs of each respective group might be used in making up the composite sample of yolks for biological assay. An effort was made to prepare composite samples of yolks, which represented an equal number of eggs obtained from the same number of birds in each of the respective groups.

Since only the yolks of the eggs were to be used in the biological assays, the eggs were broken and the yolks and the whites were carefully separated by hand. The selected yolks from each group of birds were weighed, thoroughly mixed, placed in sanitary cardboard containers, and stored at a temperature of  $-2^{\circ}$  to  $-10^{\circ}$ F. until needed in the feeding experiments.

The antirachitic potency of these samples was determined by means of biological assay, using young rats as the experimental animals. The rats used were produced in our own stock colony, and were 21 days of age and weighed approximately 40 gm. each when placed on experiment. These animals were kept in individual metal cages which were provided with raised screen bottoms and which were housed in a darkened room, from which all daylight was excluded. At the beginning of the experiment the rats were distributed among the several groups so as to eliminate possible variations that might arise as a result of litter or sex differences. Fresh distilled water and an ample supply of the Steenbock rachitogenic diet no. 2965 was kept before each animal at all times. After having received the rachitogenic diet for a period of 18 days, the test animals were found to manifest sufficiently severe rickets for assay purposes. In each case the degree of rickets manifested was verified by means of x-ray photographs.

In the first part of the investigation, the egg yolks to be tested were incorporated directly with a known weight of the antirachitic diet. It was found in subsequent assays, however, that more consistent results could be obtained by feeding the yolk separately, in three equal portions, on the first,

third, and seventh day of the curative period; consequently the latter procedure was adopted. In this procedure, the desired quantity of yolk was weighed directly into the small glazed receptacle in which it was fed. In a few cases where the quantity was too small to be weighed conveniently, an egg yolk-basal mixture was prepared in the ratio of 1:5 or 1:10, as conditions necessitated. The amount of the mixture which contained the desired amount of yolk was then weighed in three portions and fed in the usual manner.

During the 10-day curative period, the animals were weighed on the first, third, seventh, and tenth day. On the tenth day the animals were again x-rayed, killed, and 'line tested' in the usual manner.

The biological assays consisted of three parts, which were as follows:

a. Samples of egg yolks, obtained during the fall of 1933 from nine groups of yearling hens (hatched April, 1932) that were nearing the end of their first season of egg production, were assayed biologically in the above manner.

b. In order to obtain data concerning the effect of the period of egg production on the ability of the hen to transfer antirachitic potency from the diet to the egg, a second series of biological assays was conducted. The egg yolks used in this series were obtained during the spring of 1934 from eight groups of pullets (hatched November, 1933), which were receiving dietaries similar to those reported above, but which were just beginning their first season of egg production.

c. A third series of biological assays was made using egg yolks from three groups of yearling hens. One group received an optimum level of fortified cod liver oil, and the other two groups received excessive quantities of vitamin D in the form of fortified cod liver oil and viosterol (in corn oil), respectively.

The data obtained in this investigation are presented in condensed form in the following tables and figure (tables 1 to 3 and fig. 1).

In the case of the fall series, the sampling of eggs was begun on September 5th, and continued until September 15th. Since the hens used in this series were approaching the end of a season of egg production, the egg yield was irregular. This was especially true of those groups of birds which received less than the optimum amount of cod liver oil, and which were not allowed access to range. It was thought desirable that each sample be composed of a comparatively large number of egg yolks to insure a representative sample and to have sufficient material for feeding experiments. It was planned, therefore, to use the yolks from 3 dozen eggs in each sample, except in the cases of groups IV, VI, and VIII. Samples from these groups were to contain the yolks from a larger number of eggs, as these samples were to be used in the preliminary (trial) assays. However, as may be observed from table 1, such a systematic method of sampling was impossible owing to the fact that the hens comprising groups II and III produced so few eggs.

Table 1 also presents a summary of the results of the biological assays made of the nine samples of egg yolks produced by the respective groups of hens comprising the fall series. The percentage of positive line tests was computed on the basis of the number of animals which responded normally during the test period and which gave a two plus (2+)<sup>4</sup> healing when line tested. When 60 per cent or more of the test animals showed a (2+) line test, after being fed a definite amount of the egg yolk, this amount of yolk was considered the critical level.

In the preliminary feeding technic, as has been previously stated, the weighed quantities of yolk were mixed with a known weight of the antirachitic diet and fed in this manner. Owing to the fact that some of the test animals used in the earlier studies died during the 10-day curative period, due in all probability to the partial decomposition of the yolk

<sup>4</sup> An animal was rated as showing a (2+) healing when the line test showed a narrow continuous linear deposit of calcium salts on the metaphyseal side of the epiphyseal cartilage.

during the time it remained in the feed container, exposed to the warm temperature of the laboratory, the technic was modified so as to feed the yolk in small allotments, separately of the basal diet.

TABLE 1

*Showing the results of the biological assays of the samples of egg yolks produced by groups of mature hens just before reaching the end of a period of egg production*

GROUP NO.	PER CENT FORTIFIED COD LIVER OIL FED	NUMBER OF EGGS IN SAMPLE	WEIGHT OF EGGS IN GRAMS	PER CENT EGG YOLK	NUMBER OF RATS CON- SIDERED	QUANTITY OF EGG YOLK FED GM./RAT/DAY	PER CENT POSITIVE LINE TESTS	PER CENT POSITIVE X-RAYS	CRITICAL LEVEL GM./RAT/DAY	UNITS OF VITAMIN D (STENBOCK) PER GRAM OF EGG YOLK
II	$\frac{1}{32}$	14	711	33.3	6	0.7	70	...	0.7	0.14
					5	0.8	80	...		
					6	0.9	80	...		
					2	1.0	100	...		
III	$\frac{1}{16}$	10	557	32.1	2	0.5	0	0	0.7	0.14
					5	0.6	20	20		
					5	0.7	60	60		
IV	$\frac{1}{8}$	46	2459	32.7	2	0.1	0	0	0.4	0.25
					6	0.2	0	0		
					5	0.3	20	0		
					6	0.4	75	33		
V	$\frac{3}{16}$	36	1999	31.3	4	0.2	25	0	0.3	0.33
					8	0.3	75	50		
					5	0.4	60	60		
VI	$\frac{1}{4}$	59	3273	31.3	2	0.1	0	0	0.2	0.50
					10	0.2	60	50		
VII	$\frac{3}{8}$	36	2012	32.2	4	0.1	25	0	0.2	0.50
					4	0.2	75	60		
					2	0.3	100	100		
VIII	$\frac{1}{2}$	57	3113	32.3	10	0.1	60	40	0.1	1.00
					1	0.2	100	...		
					1	0.3	100	...		
					1	0.4	100	...		
IX	None (range hens)	35	1934	33.5	4	0.3	75	75	0.3	0.33
					3	0.4	100	100		
					2	0.5	100	100		
					4	0.6	100	100		
X	$\frac{1}{8}$ (range hens)	36	2011	31.4	4	0.025	50	...	0.05	2.00
					7	0.05	71	...		
					2	0.1	100	100		
					3	0.2	100	100		

The results presented in the table show that, in the case of the confined groups of birds, an increase in the amount of cod liver oil fed resulted in an increase in the antirachitic

TABLE 2

*Showing the results of the biological assays of the samples of egg yolks produced by groups of pullets at the beginning of their initial period of egg production*

GROUP NO.	PER CENT FORTIFIED COD LIVER OIL FED	NUMBER OF EGGS IN SAMPLE	WEIGHT OF EGGS IN GRAMS	PER CENT EGG YOLK	NUMBER OF EGGS CONSIDERED	QUANTITY OF EGG YOLK FED GM./HAT/DAY	PER CENT POSITIVE LINE TESTS	PER CENT POSITIVE X-RAYS	CRITICAL AMOUNT GM./HAT/DAY	UNITS OF VITAMIN D (STEWART) PER GRAM OF EGG YOLK
II	$\frac{1}{16}$	36	1402	27.1	4	0.7	25	0	0.8	0.12
					4	0.8	75	75		
					4	0.9	100	75		
III	$\frac{1}{8}$	36	1506	30.3	4	0.4	0	0	0.5	0.20
					4	0.5	75	50		
					4	0.6	100	100		
IV	$\frac{3}{16}$	36	1502	27.1	4	0.3	25	25	0.4	0.25
					4	0.4	75	50		
					4	0.5	75	75		
V	$\frac{1}{4}$	36	1464	27.5	4	0.3	0	0	0.4	0.25
					4	0.4	62.5	25		
					4	0.5	100	100		
VI	$\frac{5}{16}$	36	1556	26.0	4	0.2	0	0	0.3	0.33
					4	0.3	75	75		
					4	0.4	100	100		
VII	$\frac{3}{8}$	36	1567	26.2	4	0.2	25	25	0.3	0.33
					4	0.3	75	50		
					4	0.4	100	75		
VIII	$\frac{1}{2}$	36	1536	27.5	4	0.1	0	0	0.2	0.50
					4	0.2	75	75		
					4	0.3	100	100		
IX	1	36	1560	25.9	4	0.05	62.5	25	0.05	2.00
					2	0.075	75	50		
					4	0.1	87.5	75		
					2	0.2	100	75		

potency of the eggs produced. When less than  $\frac{1}{8}$  of 1 per cent of the fortified cod liver oil was fed (e.g., groups II and III) egg production was so poor as to make it difficult to obtain representative samples of yolks for biological assays. In fact the results indicate that  $\frac{1}{16}$  of 1 per cent of the cod liver

oil was not better than  $\frac{1}{32}$  of 1 per cent, in increasing the antirachitic potency of the egg yolks. It was interesting to note, however, that range hens which received no cod liver oil, but had access to sunlight (group IX) produced eggs equal in antirachitic potency to those produced by confined hens receiving the same diet supplemented with  $\frac{3}{16}$  of 1 per cent of cod liver oil (group V). An addition of  $\frac{1}{8}$  of 1 per cent

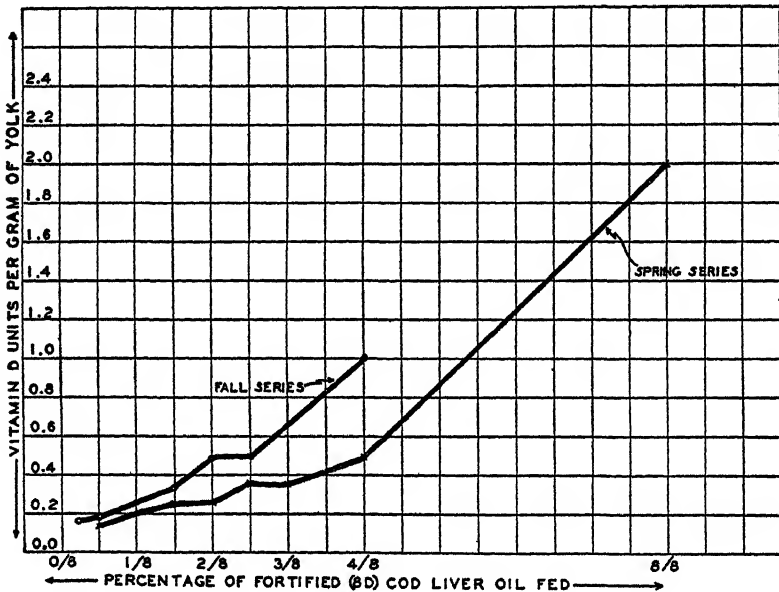


Fig. 1 Showing graphically the relationship between the percentage of fortified cod liver oil (8D) fed and the antirachitic potency of the eggs produced by the various groups of hens used in this investigation.

of cod liver oil to the diet of the range hens resulted in the production of eggs having approximately six times as much antirachitic potency as those produced by range hens receiving no cod liver oil (groups IX and X).

The relationship between the amounts of cod liver oil fed and the antirachitic potency of the eggs produced is presented graphically in figure 1.

Table 2 contains a summary of the data obtained in the second series of experiments. Since a systematic method of

sampling was possible in the case of this series, the samples consisted of two eggs from each of eighteen hens which were representative of the respective groups. It was necessary, however, to extend the collecting period for group II, 2 additional days, in order to obtain the desired number of eggs.

The vitamin D assay data found in this table were compiled in a manner similar to that described for table 1. Here, as in the first series, an increase in the amount of cod liver oil led to the production of eggs of higher antirachitic potency.

TABLE 3

*Showing the results of the biological assays of the samples of egg yolks produced by the three groups of hens, two of which received excessive doses of vitamin D, as fortified cod liver oil and viosterol, respectively*

GROUP NO.	PER CENT FORTIFIED COD LIVER OIL FED	NUMBER OF EGGS IN SAMPLE	WEIGHT OF EGGS IN GRAMS	PER CENT EGG YOLK	NUMBER OF RATS CON- SIDERED	QUANTITY OF EGG YOLK FED GM./RAT/DAY	PER CENT POSITIVE LINE TESTS	PER CENT POSITIVE X-RAYS	CRITICAL LEVEL GM./RAT/DAY	UNITS OF VITAMIN D (STEEBROCK) PER GRAM OF EGG YOLK
I (con- trol)	¼ % 8D oil	16	917	31.5	4	0.1	75	60	0.1	1.00
					8	0.2	100	62		
					8	0.3	88	88		
					4	0.4	100	100		
II (8D cod liver oil)	2 % 8D oil	24	1299	30.8	4	0.015	25	25	0.025	4.00
					7	0.025	70	57		
					4	0.035	100	100		
					4	0.05	100	100		
III (vio- sterol)	2 % 8D vio sterol	9	497	32.1	4	0.015	0	0	0.025 to 0.05	2.00 to 4.00
					8	0.025	37.5	25		
					4	0.05	100	100		
					4	0.1	100	100		

The results of the biological assays were, in general, quite comparable to the results obtained through the fall series of assays. It is to be noted, however, that for a given level of cod liver oil the pullets at the beginning of a season of egg production produced eggs which were slightly less potent, antirachitically, than were eggs produced by yearling hens at the end of a season of production. But in this particular investigation, it appears that the number of eggs produced



might have been a more important factor in determining this difference in potency than the stage of egg production.

The relationship between the amount of cod liver oil fed and the antirachitic potency of the eggs produced by the various groups of pullets comprising the spring series is also presented graphically in figure 1.

The third phase of the investigation was concerned with the effect of feeding excessive quantities of vitamin D on the antirachitic potency of the eggs produced. Summaries of the collecting and the sampling of the eggs used in this series of assays and of the results of the biological assays are presented in table 3. The data given in this table were compiled in a manner similar to that described for the other series.

Considerable difficulty was experienced in obtaining sufficient eggs to comprise a representative sample from the group of hens which received the high intake of viosterol. This difficulty, however, was not encountered with the group of hens that received an equivalent amount of vitamin D in the form of fortified cod liver oil.

From table 3 it may be observed that when the antirachitic supplement was increased eightfold, by the addition of fortified cod liver oil, there resulted a fourfold increase in the antirachitic potency of the eggs produced (groups I and II). When the increased antirachitic supplement was fed in the form of viosterol (in corn oil), the antirachitic potency of the eggs produced was somewhat less than when an equivalent unitage of cod liver oil was fed.

Since the initiation of this investigation, there have appeared in print the publications of Branion ('34), Branion, Drake, and Tisdall ('34, '35), which likewise concern the vitamin D content of eggs. While the data herein presented do not permit a direct comparison with those presented by the above investigators (due to differences in methods of technic and in manner of presenting results), it appears that the data do indicate a somewhat lower antirachitic potency than might have been reasonably expected from the findings of the above investigators.

## SUMMARY

A study has been made of the effect of the vitamin D intake of the hen on the antirachitic potency of the eggs produced. As a result of this investigation, the following conclusions are drawn:

1. The antirachitic potency of egg yolk depends on the antirachitic intake of the hen producing it.

2. The ability of the hen to transfer the antirachitic factor or factors from her diet to the egg appears to be limited.

3. When the hen's diet is supplemented with one-fourth per cent of fortified (8D) cod liver oil (which Murphy, Hunter, and Knandel ('34) found to be the optimum level when the birds were confined and denied access to direct sunlight), 1 gm. of the yolk from the eggs produced contained approximately 0.5 Steenbock unit. On this basis, an egg yolk weighing 15 gm. would contain 7.5 Steenbock units of vitamin D.

4. Yearling hens, nearing the end of a period of egg production, produced egg yolks that were more potent (antirachitically) than did pullets at the beginning of their initial period of egg production. It is suggested that this difference in potency might be due to the rate of egg production and not to the period of production.

5. At the particular level studied, the antirachitic potency of cod liver oil appeared to be more efficiently transferred from the diet to the egg yolk than was an equal unitage of viosterol.

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# QUANTITATIVE EXPERIMENTS ON THE OCCURRENCE OF VITAMIN B IN ORGANS

JESSIE B. BRODIE AND FLORENCE L. MACLEOD

*Department of Chemistry, Columbia University, New York City*

## TWO FIGURES

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Before the differentiation of vitamin B into its two factors, B(B<sub>1</sub>) and G(B<sub>2</sub>), investigations had been made to show the antineuritic and the growth-promoting effects of different organs of the body. Cooper ('12) showed that smaller amounts of sheep's brain and ox-heart were necessary to protect against polyneuritis than of beef muscle. Later Cooper ('14) concentrated, from cardiac muscle, a substance which would cure polyneuritis and Funk ('12) concentrated a similar substance from ox-brain. Eddy ('17) improved the growth of children and of rats suffering from marasmus by giving them an extract of sheep's pancreas.

Osborne and Mendel ('18) showed that 19 per cent dried pig's cardiac muscle, 22 per cent kidney, 32 per cent brain or 10 per cent liver, as the sole source of protein and water-soluble vitamin B in the diet, induced normal growth but that 5 per cent liver failed to do so. Drummond ('18) found that the protein-free extract of whole fish, as the only source of vitamin B, produced good growth, and Chick and Hume ('17) proved that the ova of fish contained the antineuritic vitamin. Hoagland and Lee ('24) discovered that dried hen's liver was of lower value for preventing polyneuritis than the liver from ox, sheep, or hog.

Many workers have reported that skeletal muscle is a poor source of vitamin B. Grijns ('01) noticed that raw meat protected fowls from polyneuritis while sterilized meat did not. Osborne and Mendel ('17) reported that 20 per cent dried lean beef in the diet as sole source of protein and of vitamin B, was not suitable for growth, that meat extract supported slight growth but that meat residue was unable to maintain weight. Hoagland ('29) found lean pork a rich source of vitamin B.

Osborne and Mendel ('23), by feeding liver from rats on a high and on a low vitamin B diet, showed the effect of diet on the vitamin content of the tissues. One hundred milligrams of dried liver daily from animals on an adequate diet, supported almost normal growth while twice this amount from animals deprived of vitamin B did not. Recently Westenbrink ('32) investigated the vitamin B content of the organs of white rats on diets of high and low vitamin B content and showed a rapid loss in the organs of the animals on deprivation of this vitamin.

Since the investigations of some of these workers indicated a storage of vitamin B in certain of the tissues of the rat, and the influence of diet thereon, it was the purpose of this investigation to determine more quantitatively the storage of vitamin B in the body of animals of known dietary history, and the influence of diet on that storage.

#### EXPERIMENTAL

*General method.* The rat growth method for the determination of vitamin B developed by Chase and Sherman ('31) in this laboratory was used. The tissues to be tested were fed to rats which had been depleted of their stores of vitamin B and were receiving a diet free from vitamin B but adequate in other respects. These tissues served as the only source of this vitamin.

*Rats used for feeding.* The rats, whose tissues were fed as sources of vitamin B were healthy young adults, reared on diet B of this laboratory which consists of whole milk powder,

one-third by weight, whole wheat, two-thirds by weight, and salt to the extent of 2 per cent of the weight of the wheat. Males and females were used alternately, care being taken not to feed the tissues of females whose bodies had been depleted by the recent bearing and suckling of young.

The animals were chloroformed as they were needed and bled by cutting the jugular vein and artery. The tissues to be tested were removed and kept on ice in glass stoppered bottles while the separate amounts were being weighed. The portions were then fed at once. These portions were weighed on small watch glasses and fed three times a week.

*Distribution of vitamin B in the body of the rat.* Quantitative determinations were made of the relative vitamin B content of muscle, liver, kidney, heart, brain, lung, spleen, and blood of adult rats which had been fed as described above. In the case of liver and muscle, young rats of the same litter were fed amounts that would give different rates of growth but the other tissues were fed at one level only. The portions of kidney, lung, brain, and spleen chosen for feeding were the largest average amounts that could be obtained from the animals. Figure 1 shows the average gains of these animals.

From the results obtained we can conclude that, in rats fed as here described, liver is gram for gram ten times as rich a source of vitamin B as is muscle. Kidney is about one-half and brain is about one-third as rich per gram as liver. Heart is almost as rich as liver and nearly ten times as rich as muscle. Spleen, lung, and blood contain only traces of the vitamin.

#### INFLUENCE OF DIET ON VITAMIN B CONTENT OF TISSUES

*A comparison of the tissues of rats on a diet rich in vitamin B with tissues from rats on a diet lacking vitamin B.* The amounts of vitamin B in the different tissues of rats reared on a normal diet (diet B) were determined by feeding the tissues to experimental animals. Using the results so obtained as standards, a comparison was made with tissues from rats on a

vitamin B free diet and with rats receiving a diet rich in vitamin B.

Young growing rats, reared to 4 months of age on diet B, were divided into two groups. One group was given a diet lacking vitamin B while another group was continued on diet B, to which had been added 2 per cent of brewers' yeast to augment the vitamin B content. When the rats, which received no vitamin B, had been on the vitamin B free diet between 4 and 5 weeks, they showed typical symptoms of vitamin B deficiency. All were emaciated, weak, had a hunched-

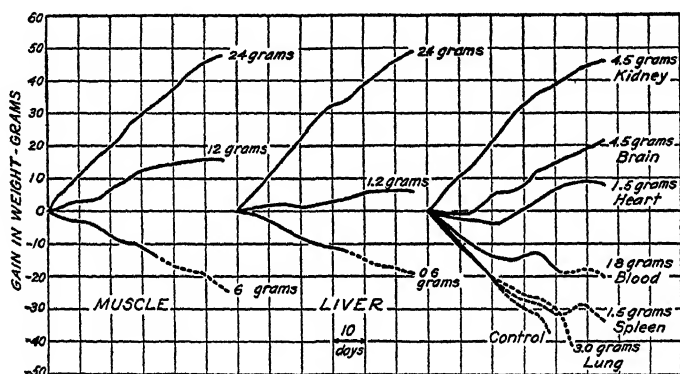


Fig. 1 Average gain curves of rats receiving a vitamin B free diet plus different amounts of tissue per week from animals reared on diet B. Broken line indicates point at which one or more rats died.

back appearance and appeared very torpid. Some had developed polyneuritis. At this time their tissues were fed to the experimental animals. At the same time, the tissues of the group which had received for the same length of time, extra vitamin B in the form of yeast added to diet B, were also fed to the experimental animals. Figure 2 shows the average gains of these animals.

The precautions stated previously were observed. The portions, except kidney, were fed at the same level as before. It was not possible to obtain as large an amount of kidney from the animals depleted of vitamin B as from normal animals.

The results obtained by feeding experimental animals the tissues of rats kept for 1 month on a diet free from vitamin B showed that there was too little vitamin B to measure in

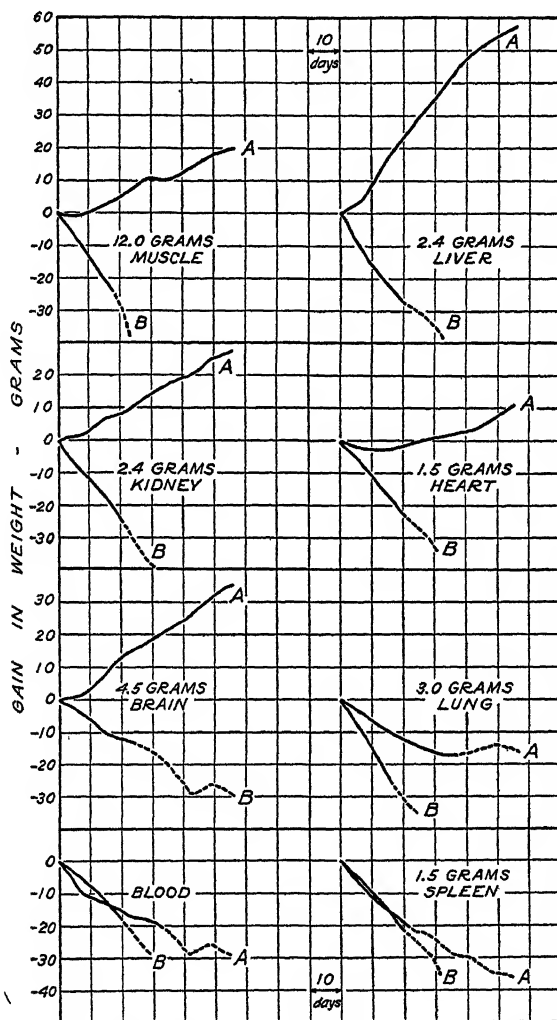


Fig. 2 A, Average gain curves of rats receiving a vitamin B free diet plus different amounts of tissue per week from animals reared on diet B plus 2 per cent yeast. B, Average gains curves of rats receiving a vitamin B free diet plus different amounts of tissue per week from animals reared on a vitamin B free diet. Broken line indicates point at which one or more rats died.



any of the tissues except brain. The animals which received brain as their supply of the vitamin did not maintain their weight but survived an average of 17.8 days longer than did the negative controls. Two animals developed severe polyneuritis. The gains and survivals of all the other experimental animals fed tissue from the rats receiving the vitamin B free diet for 1 month, approximated those of the negative controls indicating, that in all tissues except brain, there was too little vitamin B to be measured.

In the group which received tissue from the animals kept on the vitamin B rich diet for 1 month, there is evidence of a decided increase in the amount of vitamin B stored in some of the tissues. Of these tissues, muscle, liver, kidney, and brain appear to increase their store of vitamin B in about the same relative proportion, since in the case of the experimental animals receiving the tissue of animals fed a diet rich in vitamin B the average growth was from about 10 to 15 gm. more than that of the experimental animals receiving the tissue of animals reared on diet B. There was a small increase of vitamin B in the lung of the animals receiving the vitamin B rich diet. Heart, spleen and blood seem to store no extra vitamin B since the growth curves and survivals of the animals receiving heart, spleen and blood from animals reared on diet B plus yeast parallel those of the animals receiving the same tissues from animals reared on diet B.

#### SUMMARY

The distribution of vitamin B in the body of rats reared on diets containing different amounts of vitamin B was studied.

The results show that in the body of the rat reared on a normal diet, diet B of this laboratory, there is ten times as much vitamin B per gram in the liver as in the muscle. Kidney is about one-half and brain is about one-third as rich per gram as liver. Heart is almost as rich as liver and nearly ten times as rich per gram as muscle. Blood, spleen, and lung, in the amounts fed, seemed to contain only traces of the vitamin.

By comparing the growth of experimental animals which had been given tissue from animals fed for 1 month on a diet lacking vitamin B with the growth of experimental animals receiving tissue from animals kept for 1 month on a normal diet (diet B) to which had been added 2 per cent of brewers' yeast to augment the vitamin B content, it was found that the former animals, except for those receiving brain, lived no longer on an average than did the negative controls. This showed either a complete lack of vitamin B in the tissues of the depleted animals or too small an amount to be detected by this method. The animals receiving the brain tissues lived longer than did the negative controls, some even surviving the experimental period, but all lost weight rapidly. The experimental animals receiving the tissues of rats fed for 1 month on a diet containing extra yeast showed increased storage of vitamin B in some of their tissues. In the case of the experimental animals receiving muscle, liver, kidney and brain, there was an increase in growth of from 10 to 15 gm. over that made by the animals receiving tissue of rats reared on the normal diet. The experimental animals receiving lung lived an average of 12 days longer than did the experimental animals receiving the lung tissue of animals reared on the normal diet. Heart, spleen and blood showed no evidence of extra storage of vitamin B when the amount of vitamin B in the diet was increased.

It has been shown, therefore, by these experiments that the amount of vitamin B in the body of the rat may be changed within certain limits by varying the amount of vitamin B in the diet.

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# THE EFFECT OF DINITROPHENOL ON CALCIUM AND PHOSPHORUS METABOLISM

CLARENCE L. ROBBINS

*Department of Internal Medicine, Yale University, New Haven*

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Increased excretion of calcium and phosphorus characteristically accompanies the elevation of the basal metabolic rate due to hyperthyroidism but does not occur in conditions of increased metabolism due to fever or leukemia (Aub et al., '29). It has been suggested that the intrinsic metabolic rate of bone tissue may be quite independent of changes in the metabolic rate of the other tissues of the body and that the effect of the thyroid hormones on calcium metabolism is due to a specific augmentation of osteoclastic processes (Albright, Bauer and Aub, '31). The work here reported was undertaken to ascertain whether dinitrophenol might possess the property of increasing bone metabolism in addition to its demonstrated action in elevating the metabolism of entire animals (Magne, Mayer and Plantefol, '32; Tainter and Cutting, '33), surviving viscera and tissues (De Meio, Martin and Field, '34; McCord, '34) and yeasts (Field, Martin and Field, '34). Conclusions regarding bone metabolism are based on the assumption that significant changes in the rate of bone formation or destruction will probably be reflected in appreciable changes in the excretion of the chief mineral constituents of bone, calcium and phosphorus.

## METHODS

The subjects of these studies were three female patients with atrophic arthritis on the medical wards of the New Haven Hospital. In every case the arthritis was of at least

10 years duration and the clinical course had been essentially stationary for some time prior to the experimental period.<sup>1</sup> Although x-rays revealed varying degrees of bone rarefaction and overgrowth, depending on the site and duration of the joint disease in each individual case, there were no abnormalities of calcium and phosphorus metabolism apparent in the balance studies.

The general plan of the balance experiments and the technics of the collection, preparation and analyses of the excreta have been described in a previous communication (Robbins and Kydd, '35). Essentially the same diet, identical for each day of the experiments, was given to each subject. Except in the case of subject 2, who occasionally refused some food because of gastric distress, the diets were eaten completely. A 3-day fore-period to allow adjustment to the experimental diet preceded the collection of excreta in each case.

#### RESULTS

The results of the experiments are recorded in the table. In the interests of brevity only the average figures for the balances before and during the administration of dinitrophenol are presented. In case 1 the balances of calcium and phosphorus were definitely negative, while in cases 2 and 3 an identical diet resulted in positive balances. The balance of nitrogen was negative in case 1, positive in case 2 and in equilibrium in case 3. In none of the cases did the excretion of nitrogen, calcium or phosphorus change significantly when the basal metabolic rate was increased 37 per cent by the administration of dinitrophenol. The excretion of ammonia and the values for titratable acidity minus  $\text{CO}_2$  in the urine of case 1 remained unaffected by the increase in oxidative metabolism. At the beginning of the administration of dinitrophenol to case 1, there was a mild diuresis with a slight increase in urine chlorides, but thereafter the chloride excretion returned to the level observed in the control periods. A similar diuresis did not occur in the other two cases.

<sup>1</sup>The report of the clinical response of these and other patients with chronic atrophic arthritis to the administration of dinitrophenol is reserved for future publication.

*Nitrogen, calcium and phosphorus metabolism per 3-day period (dinitrophenol periods are in italics)*

CASE NO.	PERIODS	DATES	NITROGEN			CALCIUM				PHOSPHORUS				BASAL METABOLISM	WEIGHT
			Intake	Output	Balance	Intake	Urine	Stool	Balance	Intake	Urine	Stool	Balance	Date	
			gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.		kilos
1	I and II	1934 8/22-8/28	31.5	36.2	-4.7	1.89	0.70	1.56	-0.37	3.18	2.32	1.26	-0.40	8/27	55.4
	III and IV	8/28-9/3	31.5	34.6	-3.1	1.89	0.76	1.57	-0.44	3.18	2.32	1.16	-0.30	9/4	54.7
2	I and II	5/29-6/4	30.7	26.2	+4.5	1.80	0.25	1.45	+0.10	3.04	1.60	1.11	+0.33	6/4	56.9
	III and IV	6/4-6/10	30.8	25.2	+5.6	1.84	0.20	1.59	+0.25	2.99	1.38	1.04	+0.57	6/11	56.6
3	I-III	1/10-1/19	31.5	36.5	-5.0	1.89	1.86 <sup>1</sup>	1.86 <sup>1</sup>	+0.03	3.18	2.97 <sup>1</sup>	2.97 <sup>1</sup>	+0.21	1/3	77.2
	IV-VII	1/19-1/31	31.5	31.9	-0.4	1.89	1.79 <sup>1</sup>	1.79 <sup>1</sup>	+0.10	3.18	3.00 <sup>1</sup>	3.00 <sup>1</sup>	+0.18	2/3	76.6

<sup>1</sup> Total output in stool and urine mixture.

Examination of the blood serum at frequent intervals during the individual experiments revealed no changes in the normal concentrations of calcium, phosphorus and proteins of the serum.

#### DISCUSSION

Since each subject served as her own control, speculations concerning differences in the individual response to an identical diet are not germane to the general observation that dinitrophenol in doses sufficient to elevate the basal metabolic rate 37 per cent above the normal does not appear to affect the rate of excretion of calcium and phosphorus. Whether or not the actual respiratory exchange of the cellular elements of bone is increased by dinitrophenol is beyond the scope of metabolic balance studies but certainly there is no significant change in the rate of deposition or resorption of bone salts. According to the theory (Albright, Bauer and Aub, '31) which attributes osteomalacia in hyperthyroidism to a specific factor in the thyroid hormone the negative results with dinitrophenol could have been predicted.

The failure of dinitrophenol to influence nitrogen excretion is in agreement with the results of the animal experiments of Magne, Mayer and Plantefol ('32) and the clinical studies of Cutting and Tainter ('33). Although from the data here presented it is not possible to reach any final conclusion regarding the metabolic mixture during the dinitrophenol periods, it would appear that protein combustion was not increased. This is especially evident in case 1 where the negative nitrogen balance in the control periods indicates an inadequate supply of exogenous protein for the metabolic mixture even when the basal metabolic rate was 12 per cent below normal. The acceleration of metabolism to 25 per cent above normal in a period of 6 days did not increase the rate of depletion of the tissue proteins; on the contrary, there was, if anything, a tendency for the contribution of body proteins to the metabolic mixture to diminish.

## SUMMARY AND CONCLUSIONS

Calcium, phosphorus and nitrogen balances in three subjects before and during administration of dinitrophenol are reported. An increase of 37 per cent in the basal metabolic rate is accompanied by no significant change in the excretion of these elements. The excretion of ammonia and the titratable acidity minus  $\text{CO}_2$  in the urine of one subject was unaltered by the increase in oxidative metabolism due to dinitrophenol. The studies offer further evidence that increased metabolism of the body as a whole is not necessarily shared by the skeleton, at least in so far as changes in the intrinsic metabolism of bones are measurable by alterations in the total exchanges of the bone salts.

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# SUPPLEMENTARY VALUES OF ANIMAL PROTEIN CONCENTRATES IN CHICK RATIONS

H. J. ALMQUIST, E. L. R. STOKSTAD AND E. R. HALBROOK  
*University of California, Berkeley*

TWO FIGURES

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An extensive variation in the nutritive value of animal protein concentrates, when employed as supplementary sources of protein in chick rations, has been convincingly demonstrated by Prange, Carrick and Hauge ('28, '28 a, '28 b), Titus, McNally and Hillberg ('30), Allardyce, Henderson and Asmundson ('33), Johnson and Brazie ('34), Record, Bethke and Wilder ('34) and Record, Bethke, Wilder and Kennard ('34).

In experiments designed to investigate further this variation in nutritive value, similar results have been obtained which are the basis of the present report.

## PROCEDURE

The plan of research consisted in supplementing an otherwise adequate basal ration for chicks with equal levels of crude protein from various animal protein concentrates, primarily meat scraps, tankages, cracklings and similar by-products of the meat packing industry. In addition, a sample of beef, prepared in the laboratory by vacuum drying at 100°C. and then extracting most of the fat with petroleum ether, was also fed as representing the ideal meat protein concentrate. Analyses and brief descriptions of these materials are given in table 1.

In the first trial, the mixed feeds were composed of ground wheat 23 parts, ground yellow corn 56 parts, dried brewer's yeast 2 parts, salt 1 part, biologically tested sardine oil 1 part and the protein concentrate in amount sufficient to supply 6 per cent of crude animal protein. The total protein level was approximately 16 per cent. In trial II, the feeds were composed of the same ingredients in the same proportions, except that the crude animal protein level was raised to 8 per cent, the total protein level was raised to 18 per cent and the amount of yeast was increased to 3 per cent. Bone ash was added, where necessary, to bring the feeds to the same ash content. In trial III, the feeds were mixed as in trial II, except that the level of crude animal protein was 7 per cent and starch was used in place of some of the ground wheat in order to lower the vegetable protein to 9 per cent. The total protein level was 16 per cent. The mixtures were checked by analyses and adjustments made where necessary. The feeds were uniform and adequate in all respects, except for the variation in animal protein quality.

Day-old single comb White Leghorn chicks were placed in electrically heated battery brooders and given the various mixed rations and water ad. lib. The weights of the chicks and the weights of the feed consumed were determined at intervals of 2 weeks for a period of 6 weeks. The temperature of the brooders was adjusted to 95°F. for the first week and then lowered 5° per week for the remainder of the period. Special precautions were taken to avoid wastage of feed in trials II and III. Results of these feeding trials are given in table 2.

The gain per unit of feed consumed, as given in table 2, was calculated for the last 2 weeks of the growing period, rather than for the entire period, since about one-half of the total growth and total feed consumption occurred during this interval and mortality was at a minimum. Such mortality as occurred was not attributable to any specific cause and the chicks which died were considered constitutional weaklings that should not be included in growth summaries. The

feed consumed by chicks dying during the last 2 weeks was corrected for on the assumption that such chicks ate at an average rate until 1 day preceding death.

#### DISCUSSION

Brief inspection of table 2 is sufficient to show that the quality of the animal protein concentrate has a profound influence on chick growth. For example, the minimum average weight at 6 weeks in trial II was 119 gm. while the maximum was 321. In trial III, the corresponding weights were 89 and 379 gm., respectively. Since preliminary experiments indicated that 2 per cent of a dried brewer's yeast was adequate as a supplementary source of vitamin G in these rations, it is very unlikely that the growth results were influenced by a deficiency of this vitamin, especially where 3 per cent of the yeast was used. The variation in growth can fairly be attributed to the variation in nutritive value of the supplementary protein concentrates.

To some extent the nutritive values of animal protein concentrates have been investigated from the standpoint of type and condition of raw materials, methods of cooking and methods of drying and some explanations have been advanced as to the causes of variation in nutritive values.

In regard to type of raw materials, Hoagland and Snider ('26, '26 a, '27) have shown wide differences in the nutritive value of different animal organs and tissues. Mitchell and Carman ('26) and Mitchell, Beadles and Kruger ('27) demonstrated that meats containing higher amounts of connective tissue were inferior in nutritive value. Similar results have been obtained by Pittman, McCammon and Holman ('34). Meal prepared from the edible portions of fish was superior to and more digestible than meal prepared from cannery fish waste, in the experiments of Wilder, Bethke and Record ('34).

Curtis, Hauge and Kraybill ('32) showed that the hot-water-soluble fraction of tankage and similar products was inadequate, even for maintenance, while the hot-water-insoluble fraction was slightly superior to the original materials. A

similar experiment with haddock meal by Wilder, Bethke and Record ('34) gave a biological value of 34 for the hot-water-soluble fraction as compared to 97 for the insoluble fraction. Both investigations indicated that when the 'stick' or water extract was included in the final product the nutritive value was lowered.

The only protein recognizable as such in the hot-water-soluble fraction of a well-cooked animal protein concentrate is gelatin derived from the collagen of cartilage, connective tissues, skin and bone. The nutritional inadequacy of gelatin has been demonstrated by Jackson, Sommer and Rose ('28) and by Jones and Nelson ('31). These workers were unable to improve the nutritive value of gelatin by supplying the amino acids in which gelatin is known to be deficient.

Ingvaldsen ('29 a) has shown that putrefaction of the raw materials has a destructive effect on essential amino acids in fish meals and greatly increases the proportion of water-soluble nitrogen. He also found ('29) that high temperatures caused a diminution of the arginine and cystine in fish meals.

Morgan and Kern ('34) concluded that cooking caused a heat injury to meat protein which increased in severity with the length of exposure and the height of the temperature reached. Morgan ('31) and Fixsen and Jackson ('32) agree that the dry heating of casein causes a marked decline in its nutritive value. Evidence was found by Record, Bethke, Wilder and Kennard ('34) and by Record, Bethke and Wilder ('34) that higher drying temperatures adversely affected the nutritive values of fish meals, causing lowered digestibility and lowered biological value of the digestible portion.

Maynard, Bender and McKay ('32) found vacuum-dried white fish meal (cod and haddock) superior to flame-dried menhaden meal in growth promoting qualities. Maynard and Tunnison ('32) concluded that the superior nutritive value of the white fish meal was caused by the lower drying temperature and by the better quality of the raw material. Schneider ('32), who studied the same samples, showed that this superiority was due both to a higher digestibility and to a better utilization of the digestible fraction.

It is an obvious fact that the presence of undigestible protein reduces the nutritive value of a protein concentrate. According to Armsby and Moulton ('25), researches by Stuttzer in 1881, Pfeiffer in 1886 and by Kühn in 1894 showed that in vitro tests for undigestible protein gave results which agreed closely with biological tests. In other words, artificial and natural digestion of protein yielded comparable results when the latter was computed on the basis of the pepsin-insoluble nitrogen of the feces. Morgan and his associates ('09) obtained results which seemed to confirm this conclusion fully. It is reasonable to assume, therefore, that the portion of the animal protein concentrate not dissolved by prolonged treatment with pepsin at the optimum conditions for this enzyme is of negligible nutritive value.

The above literature has been cited in some detail at this point because it served as a basis for further efforts to explain the widely different nutritive values of the products used in the present tests. Although the nature and method of manufacture of most of these products was well known, there seemed little purpose in relying on manufacturers data since the product of a given plant may vary considerably. Efforts were directed toward the chemical investigation of the samples, making use of suggestions found in the work cited in an effort to detect criteria of quality which would apply in a general way to all the meat industry by-products with which this report is primarily concerned.

After preliminary trials a scheme of chemical analysis was decided upon, which included the determination of the nitrogen<sup>1</sup> precipitated by cupric ion, the nitrogen not precipitated by cupric ion but precipitated by phosphotungstic acid, the nitrogen not dissolved after prolonged digestion with pepsin, and the nitrogen soluble in hot water but precipitated by cupric ion. The scheme was purposely kept simple and limited to the determination of those forms of nitrogen which were considered of the greatest influence on nutritive value.

<sup>1</sup> The term nitrogen is here employed for simplicity to indicate the nitrogen of various groups of substances.

The results obtained by the fractionation of the nitrogen according to this scheme are given in table 1. The analytical details are given below.

*Methods used in the fractionation of the nitrogen in animal protein concentrates*

<i>Reagents</i>	
Copper sulphate solution	100 gm. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per liter
Alum solution	100 gm. $\text{NaAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ per liter
Magnesium oxide	50 gm. $\text{MgO}$ suspended in 1 liter
Hydrochloric acid	250 gm. $\text{HCl}$ in 1 liter
Pepsin solution	5 gm. pepsin in 1 liter
Phosphotungstic acid	100 gm. $\text{P}_2\text{O}_5 \cdot .24\text{WO}_3 \cdot x\text{H}_2\text{O}$ in 1 liter
Acetate buffer	Approximately 1 molal, pH approximately 5

*Procedure.* A quantity of the concentrate containing 0.5 to 1.0 gm. of crude protein was placed in a porous alundum crucible and extracted with ethyl ether for approximately 24 hours in a Bailey-Walker apparatus. It was then transferred to a 250 cc. beaker, mixed with 80 cc. of water and heated on a steam bath for 30 minutes. Five cubic centimeters of alum reagent, 25 cc. of copper sulphate solution and 50 cc. of magnesium oxide suspension were then added. Heating on a steam bath was continued for 20 minutes longer. When cool, the precipitate was filtered off and washed three times with 25 cc. of 2 per cent copper sulphate solution. The nitrogen in the precipitate was determined.

The filtrate and washings from the above were boiled for 30 minutes with a slight excess of magnesium oxide to drive off ammonia and amines, filtered, washed and made acid with hydrochloric acid to about 1 N. Ten cubic centimeters of phosphotungstic acid were added and the solution was kept in an ice box over night. More phosphotungstic acid was added if precipitation had not been complete, but a large excess was avoided. The precipitate was filtered off, using a filter aid if necessary, washed once with ice cold 1 normal hydrochloric acid containing about 1 per cent of phosphotungstic acid, and the nitrogen content of the precipitate determined.

TABLE 1  
*Analyses of animal protein concentrates*

SAMPLE NO.	DESCRIPTION	PERCENTAGE OF TOTAL NITROGEN					PERCENTAGE ORUDE PROTEIN (N x 6.25)	PERCENTAGE TRYPTOPHANE IN ORUDE PROTEIN	PERCENTAGE CYSTINE IN ORUDE PROTEIN	PROTEIN QUALITY INDEX
		Copper precipitable	Phosphoric acid precipitable <sup>1</sup>	Undigestible	Hot water soluble protein	Non-protein				
42	Casein	95.2	0.9	7.5	0.8	3.9	79.0	1.50	0.20	87.6
44	Sardine meal, steam dried	97.6	0.3	10.9	2.8	2.1	67.2	2.01	0.45	85.1
48	Dogfish meal	67.3	13.8	21.4	17.0	18.9	72.3	0.78	0.86	41.2
63	Tankage	64.6	26.3	8.6	35.2	9.1	52.5	....	....	45.4
64	Meat scrap, dry rendered	90.4	4.1	9.5	14.9	5.5	56.8	1.12	0.49	73.6
65	Cracklings	86.2	4.2	9.3	13.9	9.6	49.7	....	....	70.2
66	Cracklings	90.7	6.0	12.8	19.6	3.3	53.2	1.06	0.89	68.5
69	Tankage	52.2	36.4	10.3	16.6	11.4	48.3	0.77	0.54	46.5
70	Meat scrap, wet rendered	85.0	11.9	11.6	15.9	3.1	56.2	0.62	0.71	68.6
71	Tankage	63.8	28.8	12.1	23.1	7.4	59.4	0.83	0.54	49.3
72	Tankage	71.0	21.0	18.9	17.7	8.0	52.4	0.96	0.76	49.9
73	Beef, vacuum dried	90.8	0.9	2.6	8.5	8.3	90.8	0.72	1.10	83.4
74	Sardine meal, vacuum dried	97.1	1.2	10.2	7.2	1.7	68.4	....	....	83.1
76	Whale meat meal	86.2	7.6	2.9	2.8	6.2	75.4	1.79	0.86	84.4
78	Cracklings	92.8	4.2	6.3	13.4	3.0	50.2	0.78	0.74	80.2
79	Menhaden meal, steam dried	93.0	0.8	8.3	4.0	6.2	63.0	1.87	0.96	82.6
81	Meat scrap, dry rendered	80.8	15.1	12.0	17.5	4.1	58.2	1.30	0.82	64.3
82	Sardine meal, steam dried	99.3	0.8	9.4	2.9	0.0	69.0	2.12	0.45	88.5
84	Gelatin	100.0	0.0	0.0	100.0	0.0	98.7	....	....	40.0
85	Mixture of 82 and 78							....	....	84.3

<sup>1</sup> Obtained from the filtrate from the copper precipitation.



The undigestible protein was determined on a fresh 2 gm. sample which was extracted with ethyl ether as before and transferred to a 500 cc. shaking bottle. Two hundred and fifty cubic centimeters of water, 5 cc. of hydrochloric acid and 25 cc. of freshly prepared pepsin solution were added. The mixture was shaken for 25 hours at a temperature of 37°C. Then 5 cc. of hydrochloric acid were added and the mixture was allowed to stand for 24 hours more at a temperature of 37°C. The insoluble material was filtered and washed two or three times with hot water. To obtain clear filtrates a filter aid was occasionally necessary. The nitrogen in the undissolved residue was determined.

The hot-water-soluble protein was determined by extracting a 2 gm. sample with ethyl ether until most of the fat was removed, then boiling with 200 cc. of water containing 5 cc. of the acetate buffer for 30 minutes. The mixture was filtered while hot and washed several times with hot water. The filtrate and washings were treated with cupric ion and magnesium oxide as already described, and the nitrogen content of the copper precipitate determined.

*The copper precipitable nitrogen.* Cupric ion in the presence of dilute hydroxide ion is one of the most specific reagents for intact protein. The fraction of the total protein which is precipitated by copper decreases regularly as the degree of decomposition of the protein increases. However, peptones, peptides and amino acids present among the protein degradation products are also precipitated to some extent by copper. In the laboratory, steam, acid or enzymic hydrolysis of protein concentrates was found to cause a marked lowering of the copper-precipitable nitrogen.

The percentage of the total nitrogen which was precipitable by copper varied from 52.2 to 100 in the protein concentrates. It was particularly low in those concentrates, such as tankages, prepared by wet rendering processes and other processes allowing extensive decomposition.

*The phosphotungstic acid precipitable nitrogen.* The nitrogen not precipitated by copper cannot be regarded in all cases

as having no nutritive value. In order to estimate the portion of this nitrogen to which a nutritive value may be ascribed, phosphotungstic acid was employed as a precipitant for the peptones, peptides, and amino acids which escaped the copper treatment. These phosphotungstic acid precipitates were investigated by means of the formaldehyde titration. It was found that they gave comparatively large formaldehyde titration values which were increased five- to twelvefold by acid hydrolysis indicating an average composition corresponding to that of a complex peptide. After acid hydrolysis, 45 to 65 per cent of the nitrogen in these fractions was present in the form of alpha-amino nitrogen. It is evident, therefore, that such material must have a certain nutritive value since it contains a high proportion of amino acids, especially basic amino acids which are essential to nutrition. It may be seen from table 1 that as the percentage of copper-precipitable nitrogen decreased, the percentage of nitrogen precipitated by phosphotungstic acid tended to increase. This fraction, consisting mostly of peptones, peptides and amino acids resulting from protein disintegration, varied from 0.3 to 36.4 per cent. The material not precipitated by copper or by phosphotungstic acid rarely showed more than a slight increase in the formaldehyde titration after acid hydrolysis. Hence, it may be assumed that this non-precipitable nitrogen has a negligible nutritive value, although certain amino acids may be present in small quantities.

*The undigestible nitrogen.* Nitrogen in the form of undigestible protein or other insoluble nitrogenous residues was estimated by the failure of such nitrogen to dissolve when treated with pepsin and hydrochloric acid under the optimum conditions for this enzyme. This nitrogen is automatically included in the copper precipitable nitrogen and must be separately estimated since it may be present in considerable amounts.

A further treatment of the pepsin-insoluble residues with trypsin gave no appreciably greater degree of solution. Kühn in 1894 (cited by Armsby and Moulton, '25) found that

the prolonged treatment with pepsin was equivalent to the treatment with pepsin followed by trypsin. It was apparent that the pepsin treatment alone gave the minimum value for undigestible nitrogen, hence the supplementary action of trypsin was not employed. Materials which contribute to the undigestible fraction are the keratins of hoof, horn and hair, and protein material which has been extensively denatured by high temperatures. Values for undigestible nitrogen obtained from the animal protein concentrates varied from 0.0 to 21.4 per cent of the total nitrogen.

*The hot-water-soluble protein nitrogen.* The chief hot-water-soluble protein present in animal protein concentrates is gelatin. Its low nutritive value makes necessary its separate estimation apart from other proteins in the copper precipitable fraction. In materials made largely from bone, cartilage, connective tissue and skin, the gelatin content may be very high. While such products may show a high proportion of copper-precipitable nitrogen and a low proportion of undigestible nitrogen, their nutritive values remain relatively low.

Nitrogen in this form varied, in the commercial concentrates, from 0.8 to 35.2 per cent of the total nitrogen and reached a value of 100 in the case of the gelatin sample.

*The cystine and tryptophane content.* Analyses for cystine and tryptophane were made, using the methods of Ingvaldsen ('29), Baernstein ('30) and Folin and Ciocalteu ('27), in order to determine what relation to nutritive value such analyses may bear when applied to commercial concentrates. Since cystine and tryptophane are among the most labile of the essential amino acids, they are very likely to be partially or completely destroyed by putrefaction or adverse manufacturing conditions.

The percentage of tryptophane in the crude protein varied from 0.62 to 2.12. Cystine varied from 0.20 to 1.10 per cent of the crude protein. Both are practically absent in gelatin. No attempt was made to determine what proportions of these amino acids were in the undigestible fraction. Keratins, the

undigestible proteins of hoof, horn and hair, are especially rich in cystine, and are among those substances likely to be present in the undigestible fraction.

*Analytical characteristics of various classes of concentrates.* As a class, the tankages (samples 63, 69, 71 and 72) showed the lowest copper-precipitable fraction, the highest phosphotungstic-acid-precipitable fraction, the highest undigestible fraction, and the highest hot-water-soluble fraction. The dog-fish meal (sample 48) may also be classed with these from the analytical standpoint.

The meat scraps (samples 64, 70 and 81) showed little marked difference as a class from the cracklings (samples 65, 66 and 78). These groups appeared definitely superior to the tankages in regard to the above analytical characteristics.

The vacuum-dried beef (sample 73) and the whale meat meal (sample 76) gave the most favorable analyses of the meat products. The fish meals included in the test gave results which were equally good, with the exception of the dog-fish meal. However, the investigation at the present stage has not included a sufficient number of samples of fish meals to warrant final comparisons in this report.

It may be pointed out that the method of analysis employed provides strong evidence of the condition and type of raw materials and of the effect of the manufacturing process on the quality of the product. Decomposition caused by putrefaction, autolysis or poor methods of manufacture is reflected in a decrease of the copper-precipitable-fraction and an increase in the phosphotungstic-acid-precipitable fraction. The inclusion of undigestible nitrogenous substances and the effect of excessive temperatures introduce an unduly large undigestible fraction. An increase in the cartilage, connective tissue, skin and bone content causes a corresponding increase in the hot-water-soluble protein nitrogen. The same is true when the 'stick' is included in the final product. Finally, the presence of much nitrogen in the form of ammonia, amines, urea and similar types of non-protein nitrogen causes a wide discrepancy to appear between the summation of the nitrogen

accounted for by the copper and phosphotungstic acid treatments and the total nitrogen.

It should also be pointed out that the analytical scheme which was employed does not detect the presence of toxic substances. Mortality, the only criterion of toxicity which was available in these experiments, was very low during the last 2 weeks of the trials and actually lower in the slow growing groups than in the faster growing groups, hence it would appear that toxicity was not an important factor in the growth results herein reported.

#### *Relation of analytical data to growth data*

In comparing the average weights of chicks at 6 weeks with the percentages of total nitrogen precipitated by copper, it was observed that this analytical characteristic showed a moderately good correlation with nutritive value. Samples of low nutritive value invariably had a low percentage of copper-precipitable nitrogen, while samples of high value had a high percentage. However, a high percentage of copper-precipitable nitrogen does not assure a high nutritive value, because of the possible inclusion of undigestible nitrogen and of the nitrogen of gelatin. On the other hand, a low percentage of copper-precipitable nitrogen is an excellent criterion of low nutritive value.

Proceeding a step farther, it was noted that subtraction of the undigestible nitrogen from the copper-precipitable nitrogen gave a numerical expression of quality which showed a distinctly better correlation with growth, especially among the concentrates of lower quality. This result suggested that, if the phosphotungstic-acid-precipitable nitrogen and the hot-water-soluble protein nitrogen could also be combined into the numerical expression of protein quality, the agreement of the analytical and biological results might be further improved. Since both of these fractions must be assumed to possess a lower nutritive value than corresponding quantities of undecomposed meat protein, the chief problem appeared to be the choice of a fractional factor which would express their low

value. By a process of trial it was found that, when both of these low value fractions were assigned a factor of four-tenths, the best agreement of analytical and biological results was obtained. We have on this basis, calculated for each protein concentrate an expression called the protein quality index which is based on the analytical results given in table 1. The formula for this index is:

$$\text{Protein quality index} = A - (B + .6 C) + .4 D$$

where A = Percentage of total nitrogen precipitated by copper (inclusive of B and C).

B = Percentage of total nitrogen not digestible.

C = Percentage of total nitrogen as hot-water-soluble protein.

D = Percentage of total nitrogen precipitated by phosphotungstic acid.

The values of this index for each protein concentrate are given in table 1. These indices varied from 40.0 to 88.5. On this basis, none of the concentrates represented a perfect animal protein, as such a protein would have an index of 100.

In table 2 it may be seen that the protein quality index shows a close agreement with both the total growth and the gain per unit of feed consumed during the last 2 weeks of the period. Perhaps the best way in which to express this agreement between sets of data obtained by such widely different methods is the graphical method. Figures 1 and 2 present a graphical comparison of total growth and growth per unit of feed consumed with the protein quality indices of the concentrates.

Within the limits of the expected variation, samples retained their same relative positions whether plotted on the basis of total growth or on the basis of growth per unit of feed consumed. Exceptions to this rule seemed to occur in the cases of samples 70, 78 and 85 which lagged in total growth produced but fell closely into line in respect to growth produced per unit of feed consumed during the last 2 weeks. It is possible that such results might be caused by unpalatability or any other factor causing lowered feed consumption.

Sample 85, which was composed of equal parts of protein from samples 78 and 82, gave results for total growth and growth per unit of feed consumed which were intermediate

between those obtained from 78 and 82 (cracklings and sardine meal, respectively). This result is in agreement with the report of Record, Bethke, Wilder and Kennard ('34).

TABLE 2  
*Growth data of trials I, II and III*

SAMPLE NO.	PROTEIN QUALITY INDEX	NUMBER OF CHICKS AT 6 WEEKS	AVERAGE WEIGHT OF CHICKS AT 6 WEEKS IN GRAMS	AVERAGE GAIN PER UNIT OF FEED, LAST 2 WEEKS
Trial I				
42	87.6	13	257.0	
63	45.4	13	172.0	
64	73.6	13	240.7	
65	70.2	12	230.0	
66	68.5	8	232.1	
69	46.5	11	151.9	
70	68.6	12	175.2	
71	49.3	12	162.0	
72	49.9	12	135.5	
Trial II				
42	87.6	15	269.0	0.351
44	85.1	12	321.0	0.346
48	41.2	13	119.0	0.157
64	73.6	14	262.8	.... <sup>1</sup>
66	68.5	13	229.5	0.269
69	46.5	13	144.7	0.181
70	68.6	11	200.2	0.292
71	49.3	15	169.5	0.235
72	49.9	13	141.2	0.150
73	83.4	14	278.5	0.349
Trial III				
70	68.6	14	231.5	0.322
71	49.3	21	167.3	0.244
72	49.9	14	168.5	0.262
76	84.4	10	377.0	0.370
78	80.2	28	230.8	0.337
79	82.6	13	363.0	0.389
81	64.3	15	255.9	0.336
82	88.5	23	379.0	0.369
84	40.0	11	89.0	.... <sup>1</sup>
85	84.3	20	289.8	0.357

<sup>1</sup> Unknown quantity of feed lost.

It is evident that chemical analyses such as have been described are capable of throwing much light on the variation of protein quality, one of the major problems in animal nutrition. Further investigation and application of chemical methods may lead to greater refinement and improved signifi-

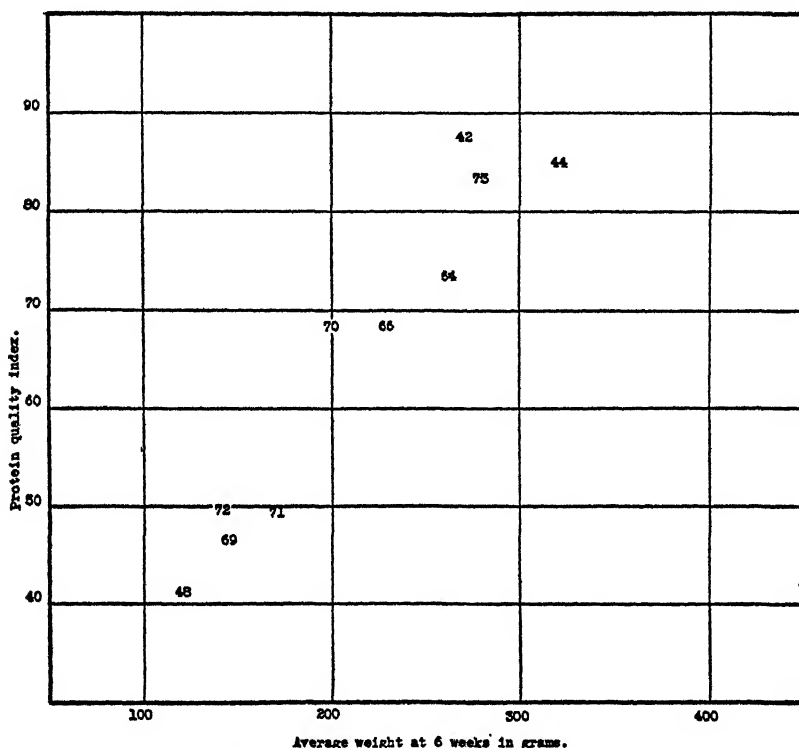


Fig.1 Total average weight at 6 weeks obtained in trial I, plotted against protein quality index. Plotted numbers refer to animal protein samples as in table 1.

cance of results. We have hesitated, however, to complicate the procedure beyond the bounds of practical applicability, preferring to ascertain first whether the simplest technic will provide a sufficiently accurate result for the purpose. It is believed that the procedure employed is the simplest which conforms to theoretical requirements.



It is very difficult to interpret amino acid levels in the complex animal and vegetable protein mixtures required by chicks. Partial deficiencies of amino acids may, to a large extent, be rectified by feeding higher levels of protein or by feeding supplementary sources of these amino acids. Moreover, the digestibility and the state of disintegration of the

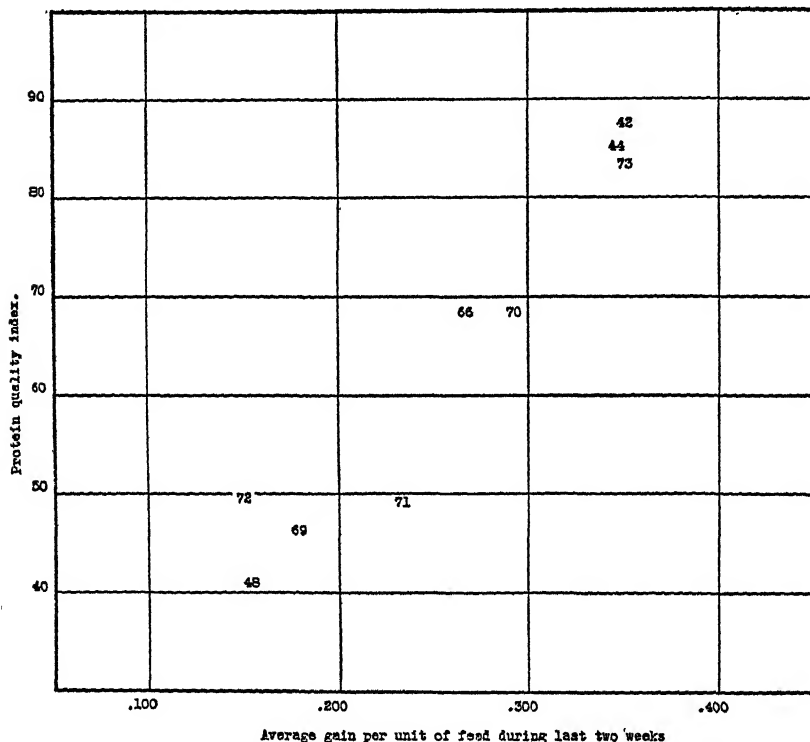


Fig. 2 Total average weight at 6 weeks obtained in trial II plotted against protein quality index.

protein may exert a large effect on its nutritive value apart from its amino acid composition.

The cystine and tryptophane content of the animal protein concentrates could not be used to predict their nutritive values. Sample 48 which gave very poor growth contained more cystine and tryptophane than did several other samples which greatly exceeded it in nutritive value. Sample 81 was

well supplied with both cystine and tryptophane and yet gave inferior results. The fact that casein is especially low in cystine seems to have had no influence on its excellent growth-promoting action.

As a further index to protein decomposition, the samples were tested for the presence of hydrogen sulphide by mixing them with dilute sulphuric acid and allowing them to stand at room temperature for 24 hours in a flask stoppered with a cork from which was suspended a moist strip of lead acetate test paper. Although considerable darkening of the test paper was obtained in many instances, no correlation was observed between the degree of darkening and the nutritive values of the protein concentrates.

#### SUMMARY

When used as supplementary sources of protein in chick growing rations, tankages gave decidedly inferior results, meat scraps and cracklings gave much better but yet unsatisfactory results, while vacuum-dried beef and whale meat meal gave very satisfactory results.

Analyses of animal protein concentrates were made for the estimation of intact protein, protein decomposition products, undigestible protein and hot-water-soluble protein. These analytical characteristics were found to exhibit a good correlation with nutritive value for chicks, when assigned the relative values of 100, 40, 0 and 40, respectively. Such analytical methods offer a possibility for the rapid laboratory determination of protein quality in commercial concentrates.

The cystine, tryptophane and hydrogen sulphide content of the protein concentrates could not be used as a basis for predicting their nutritive values.

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# A NEW TOXICANT OCCURRING NATURALLY IN CERTAIN SAMPLES OF PLANT FOODSTUFFS<sup>1</sup>

## IX. TOXIC EFFECTS OF ORALLY INGESTED SELENIUM

KURT W. FRANKE AND VAN R. POTTER

*Department of Experiment Station Chemistry, South Dakota State College,  
Brookings*

### FIVE FIGURES

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The presence of selenium (Robinson, '33) in the toxic grain used in the experiments described by Franke ('34 b) made it desirable to investigate the physiological effects of selenium compounds. The occurrence of selenium in foodstuffs gives a new aspect to the toxicology of the element. In the past the physiological effects of selenium have been studied by injection methods in the majority of cases. The authors are not aware of any published work which has involved the ad lib. feeding of artificial selenium-containing diets.

The work reported in this paper was planned in order to compare the effects produced by artificial selenium-containing diets and the effects produced by the affected natural foodstuffs which have previously been described by the authors. At present considerable attention is being focused on 'the selenium problem,' and there may be a tendency to regard selenium as the sole causative factor in the production of the natural plant toxicants. Although the present paper will

<sup>1</sup>Published with the permission of the director of the South Dakota Agricultural Experiment Station as communication no. 12 from the Department of Experiment Station Chemistry. These investigations are being carried out under the Purnell Fund and with the cooperation of the Bureau of Chemistry and Soils, Bureau of Plant Industry, Bureau of Animal Industry, and Bureau of Home Economics of the United States Department of Agriculture.

point out a number of similar physiological effects produced by the natural toxicant and the element selenium, it has not definitely been proved that the toxicity of cereal grains is strictly proportional to their selenium content.

The toxicity of selenium depends upon the ionic combination in which it is found. Thus, elemental selenium, in the form of the powdered metal, seems to be virtually harmless when added to control wheat in concentrations comparable to the amount present in toxic wheat. At the same level, sodium selenide produces marked growth depression, but no deaths occur. When fed in the form of sodium selenite or selenate, selenium produces toxic effects approaching those of the natural toxicants. Sodium selenite has probably been investigated more frequently than any other form of selenium (Jones, '09; Cathcart and Orr, '14; Levine and Flaherty, '26).

#### EXPERIMENTAL

In the present experiment, various amounts of sodium selenite were added to control wheat diets which were then fed ad lib. to white rats to determine the effect on the growth, food intake, hemoglobin, and gross pathology. Stained smears of the blood were also examined at frequent intervals.

The rats used were of Wistar Institute origin. They had been weaned at 21 days and maintained for 1 week on McCollum's diet I as described by Burr and Burr ('29). They were then divided into four groups of nine rats each, and placed in the individual drawer cages described by Franke and Franke ('34), and given diets as follows:

Group 1 received control wheat diet. Groups 2, 3, and 4 received the control wheat diet plus 22.3, 33.5, and 52.1 parts per million, respectively, of selenium in the form of sodium selenite.

The composition of the diet used is described in a former publication (Franke and Potter, '34). Selenium was added by pipetting the proper amount of a solution of sodium selenite<sup>2</sup> (2.976 mg. Se per cubic centimeter) directly into a

<sup>2</sup> C. P. obtained from Eimer and Amend.

pan containing 1 kilo of diet which was then dried, at room temperature, and thoroughly mixed.

Fresh distilled water containing a trace of iodine was available to the rats at all times.

The rats were weighed at 5-day intervals, and their food intake was recorded daily.

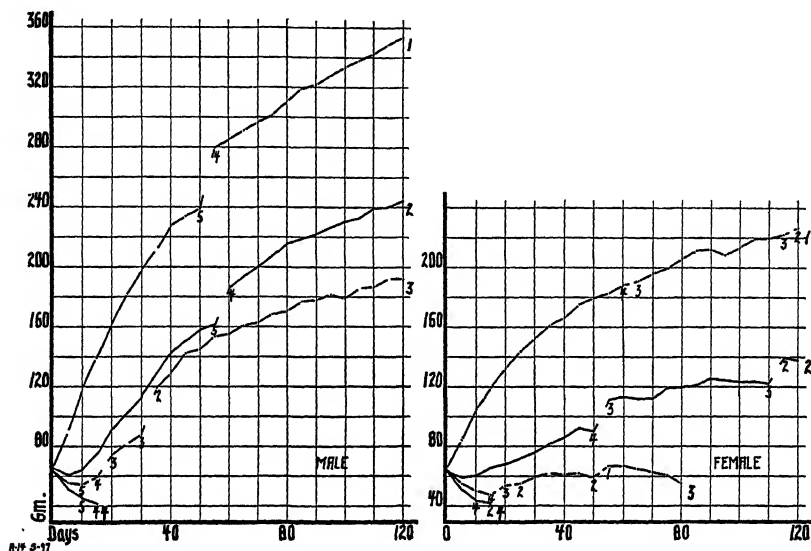


Fig. 1 Growth curves for males and females on following diets: 1) Control wheat; 2) control wheat + 22.3 parts per million of selenium as sodium selenite; 3) control wheat + 33.5 parts per million of selenium as sodium selenite; 4) control wheat + 52.1 parts per million of selenium as sodium selenite. Figures under curves indicate number of surviving animals.

Hemoglobin was determined, as in the previous paper, with greater intervals between bleedings as the experiment progressed. Blood smears were made from the same samples which were used for hemoglobin determinations and treated<sup>3</sup> with Wright's stain.<sup>4</sup>

<sup>3</sup> It was found convenient to add stain and buffer from two 10-cc. syringes, using 0.8 cc. of each solution per slide.

<sup>4</sup> Osgood and Haskins, Laboratory Diagnosis, 1931.



## DISCUSSION OF RESULTS

The experiment was terminated 359 days after it had begun. At this time there were four survivors on the 22.3 p.p.m. diet, and only one rat on the 33.5 p.p.m. diet. Three of the control animals were sacrificed during the experiment. The animals which received 52.1 p.p.m. had all died by the seventeenth day of the experiment. The growth of the rats in the various groups for the first 120 days is shown in figure 1. The growth

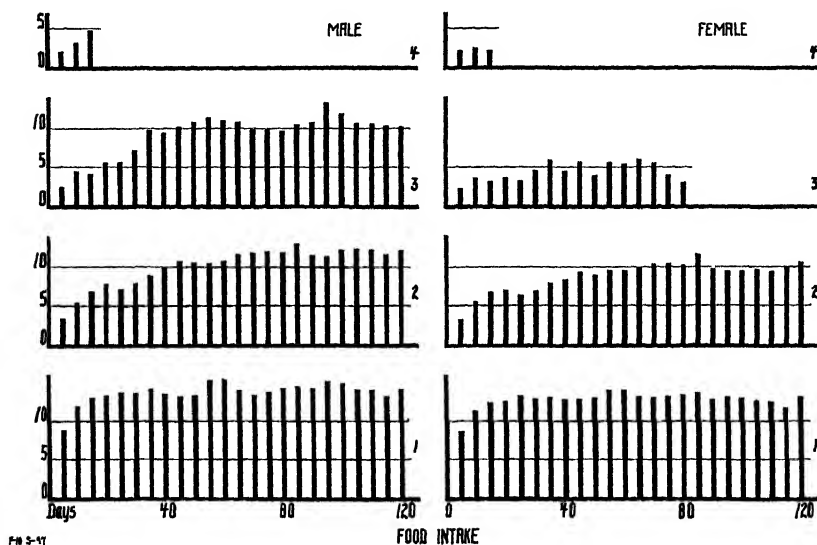


Fig. 2 Food intake per rat per day. Five-day averages. Groups numbered as in figure 1. Figures based on surviving animals as in figure 1.

is probably a direct result of the voluntary restriction of food intake, as was found to be the case with the natural toxicant (Franke and Potter, '34). The food consumption for the various groups is shown in figure 2. The food intake bears an inverse relation to the selenium content. In an experiment in which rats were given a choice between diets having various concentrations of selenium, it was found that the animals invariably chose the least toxic diet. They were apparently able to taste the selenium in the diets. However, it seems unlikely that a disagreeable taste could account for

the degree of inanition which occurred in group 4. In support of the latter idea is the fact that anorexia has been reported in nearly every instance where selenite has been injected. In a case in this laboratory, a rat fasted completely for 13 days following an injection of sodium selenite (3.7 mg. per kilo).

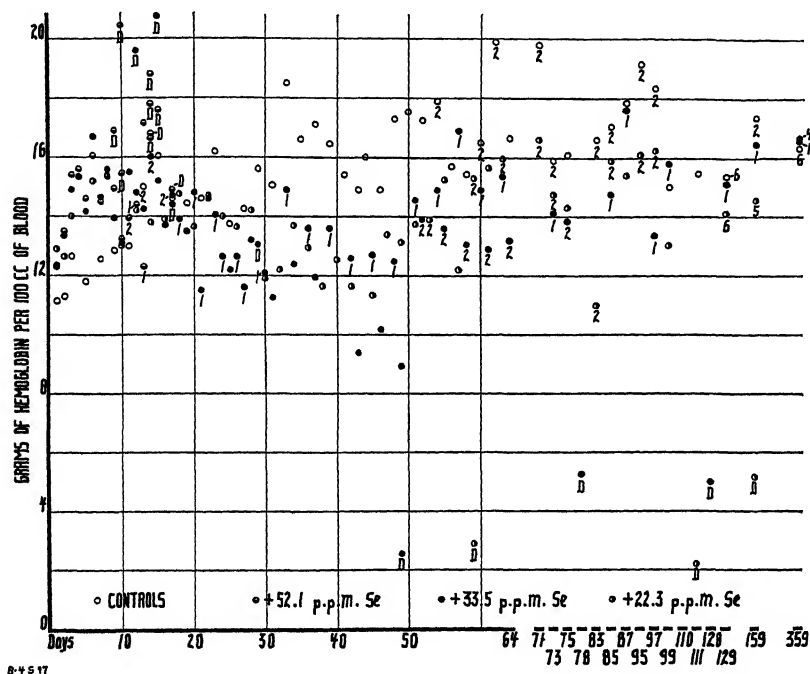


Fig. 3 Hemoglobin levels for the experimental period. Each point represents three rats unless otherwise indicated by sub-figures. The final hemoglobin levels reached by individual rats are indicated by D.

The hemoglobin levels (fig. 3) attained by the rats in the various groups show two distinct trends. Prior to a certain critical period (the ninth to seventeenth day of the experiment) the experimental rats show abnormally high levels of hemoglobin. After that date there is a distinct tendency toward anemia. High levels of hemoglobin are probably a characteristic symptom of acute selenium poisoning. The absolute amount of hemoglobin is almost certainly not increased since the blood is probably anhydremic. In this

laboratory, Painter (unpublished data) observed hemoglobin levels as high as 25 gm. per 100 cc. of blood in rats which

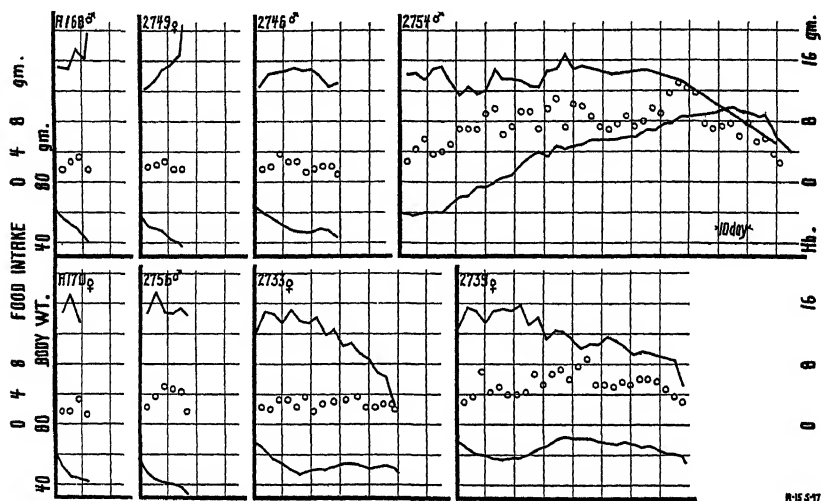


Fig. 4 Hemoglobin, growth, and food intake of individual rats in group 3. (33.5 p.p.m. Se as  $\text{Na}_2\text{SeO}_3$ ).

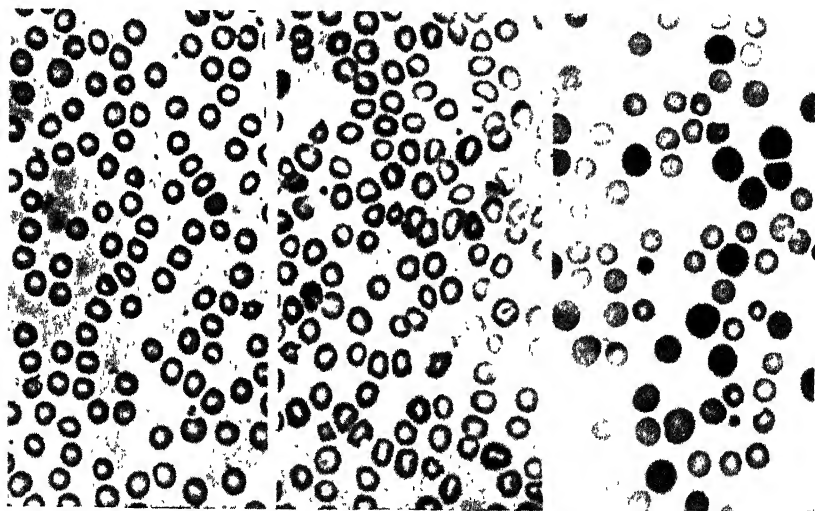


Fig. A Left: erythrocytes from a control rat (M 22); middle: erythrocytes with achromia (M 42); right: anisocytosis and polychromatophilic macrocytes (M 46). Magnified 500 diameters. E-22 orange color filter. Orthochromatic film.

were injected with lethal doses of sodium selenite. Post mortems on injected rats reveal conditions which are almost identical with those found in the rats in this series which died with high hemoglobin levels. The anhydremia can be explained in a majority of cases by the presence of considerable fluid in either the thoracic or the abdominal cavities, or in both.

Figure 3 shows that all (nine) of the rats on 52.1 p.p.m., four of the rats on 33.5 p.p.m., and none of the rats on 22.3 p.p.m. died during the so-called critical period. Most of these rats had extremely high levels of hemoglobin, and all of the rats showed acute symptoms at the post mortem.

Figure 4 shows the hemoglobin, growth and food intake for individual rats in group 3 (33.5 p.p.m.). This set of curves compares quite closely with those obtained by Franke and Potter ('34) in which the natural toxicant was used. It is easily seen, however, that the effects produced in this group were less severe than the effects produced in series 87 in the preceding paper. This is well illustrated by the fact that in the former series the food intake per rat per day was only 2.9 gm., while in this group it was 5.1 gm.

After the critical period a number of rats began to show decreases in hemoglobin and eventually developed a fatal anemia. Blood smears made throughout the course of the experiment frequently showed achromia, and later anisocytosis with polychromatophilic macrocytes (fig. A), in the anemic rats. Among the acute cases there was a slight anisocytosis with a tendency toward leucopenia. The latter symptom was observed by Jones ('12) in acute cases after injection.

Some of the rats seemed practically unaffected by the toxic diets, both from the standpoint of hemoglobin and body weight. These rats included one male on the 33.5 p.p.m. diet, and three males and two females on the 22.3 p.p.m. diet. These rats were tested for fertility by various cross matings. The only matings which proved fertile were those in which

either the male or the female was a control animal. Under these conditions, the males on 33.5 p.p.m. were able to reproduce, and one of the females cast a litter. The results seem to bear out the conclusions of Macomber ('23) in regard to the 'fertility threshold.' It is quite possible that the fertility of the experimental rats was lowered to such an extent that fertile matings could result only with control rats of high fertility. More work will need to be done on this point before definite conclusions can be reached.

The pathological symptoms readily classify into two types which are strictly analogous to those described by Franke ('34a) as subacute and chronic. The subacute cases have practically the same post mortem appearance as rats which have been injected with a lethal dose of selenium, except that they are always emaciated. The surviving rats were killed on the three hundred and fifty-ninth day, and although their hemoglobin was normal, the livers had the characteristic hob-nailed appearance which is caused by necrosis and regeneration. The case of one of the females on 33.5 p.p.m. was unique. This animal died in the latter part of the experiment with what might be considered acute symptoms. The liver showed typical lesions, but the animal was distinctly not anemic. The thorax was filled with a transudate, and the lungs were completely atelectatic. Terminal hemoglobin was not determined.

In the present investigation the sodium selenite used was assumed to be in the form of the anhydrous salt. By reducing the selenite to its elemental form, it was possible to recover 98.2 per cent of the theoretical amount of selenium. Marked differences in the solubility of samples of sodium selenite have been found, and there is some evidence that variations in toxicity may occur.

It is possible that a portion of the added selenite was reduced by contact with the diet. The authors have been unable completely to prove that no reduction occurred. However, it has been shown that elemental selenium is formed when sodium selenite is reduced and that at this level elemental

selenium is non-toxic. The toxicity of the diets containing selenite indicates per se that any reduction which occurred must have been slight.

#### CONCLUSION

The symptoms of selenium poisoning, produced by feeding small quantities of sodium selenite, in an otherwise normal diet, are virtually identical with the symptoms produced by the natural plant toxicant described by the authors elsewhere (Franke, '34 a; Franke and Potter, '34). Symptoms considered are 1) growth, 2) food intake, 3) hemoglobin levels, 4) gross pathology. It is realized that these factors alone are far from conclusive. They do, however, support the idea that selenium is very closely connected with the natural toxicant.

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# A NEW TOXICANT OCCURRING NATURALLY IN CERTAIN SAMPLES OF PLANT FOODSTUFFS<sup>1</sup>

## X. THE EFFECT OF FEEDING TOXIC FOODSTUFFS IN VARYING AMOUNTS, AND FOR DIFFERENT TIME PERIODS

KURT W. FRANKE

*Department of Experiment Station Chemistry, South Dakota State College,  
Brookings*

FOUR FIGURES

(Received for publication March 8, 1935)

Two series of feeding experiments were carried out on rats: the first series was fed varying amounts of toxic corn in the diet, and the second series was fed a diet containing toxic wheat. These series were intended to simulate: 1) the feeding of individual samples of varying degrees of toxicity occurring in naturally grown foodstuffs (Franke, '34), and 2) the effect on the growth of animals by changing the diet containing toxic grain to one containing control grain.

### *Feeding varying amounts of toxic corn in the diet*

The rats used in these two series were of Wistar Institute origin and had been weaned at 21 days of age and maintained for 1 week on a stock diet. Litter mates were then divided into the required number of groups of ten rats each so that each group contained the same number of males and females, and weighed approximately the same. The rats were weighed every 5 days and the daily food intake recorded.

<sup>1</sup> Published with the permission of the director of the South Dakota Agricultural Experiment Station as communication no. 14 from the Department of Experiment Station Chemistry. These investigations are being carried out under the Purnell Fund and with the cooperation of the Bureau of Chemistry and Soils, Bureau of Plant Industry, Bureau of Animal Industry, and Bureau of Home Economics of the United States Department of Agriculture.



The composition of the diet was as follows:

	<i>Per cent</i>
Ground whole corn	70.0
Casein	11.0
Sucrose	15.0
Lard	2.0
Calcium carbonate	1.4
Sodium chloride	0.6
Total	100.0

Each rat was kept in an individual cage as described by Burr and Burr ('29). The diets of the five groups of ten rats each varied in the corn content as follows:

<i>Group</i>	<i>Per cent of corn fraction of diet</i>	<i>Per cent of total diet</i>
A	Toxic corn lab. no. 388 0.0	0.0
B	Toxic corn lab. no. 388 25.0	17.5
C	Toxic corn lab. no. 388 50.0	35.0
D	Toxic corn lab. no. 388 75.0	52.5
E	Toxic corn lab. no. 388 100.0	70.0

The average growth of these groups is given for the males in figure 1, and for the females in figure 2. It is of interest to note that group B, which received only 17.5 per cent of toxic corn lab. no. 388, had a decided lower growth than control group A, and that two out of ten rats had died by the one hundred and fiftieth day of experimentation. The growth of group C, receiving 35 per cent toxic corn, was still lower, and four of the ten animals died. Where the diet contained 52.5 per cent toxic corn (group D), all the rats had died by the one hundred and twenty-third day, and in group E, receiving 70 per cent toxic corn, all had died by the ninetieth day.

The external appearance of the rats in group B did not differ much from those in group A except for size, but in the other groups decided differences were noted. All gradations from normal to extremely abnormal animals, both as to growth and pathology, were noted as described before (Franke, l.c.).

As the per cent of toxic foodstuff in the diet increased, the food consumption decreased. The average food consumption for group A was 13.16 gm.; B, 10.46 gm.; C, 8.21 gm.; D, 5.92 gm.; and E, 4.74 gm. per rat day to the ninetieth day. As

mentioned in previous reports, the decreased growth correlates with the decreased food consumption, but the decreased food consumption, deaths and pathological lesions are inherent to the toxicant present.

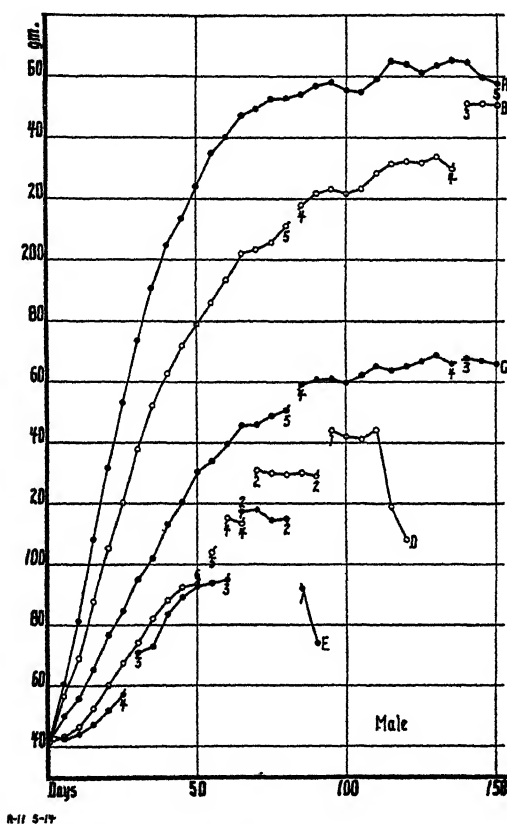


Fig. 1 Average growth curves of males obtaining: group A, toxic corn lab. no. 388, 0.0 per cent of diet; group B, toxic corn lab. no. 388, 17.5 per cent of diet; group C, toxic corn lab. no. 388, 35.0 per cent of diet; group D, toxic corn lab. no. 388, 52.5 per cent of diet; group E, toxic corn lab. no. 388, 70.0 per cent of diet (the sub-numerals indicate the number of animals averaged).

*The preliminary feeding of toxic foodstuffs followed by control foodstuffs*

Forty rats were divided into four groups and placed in individual drawer cages (Franke and Franke, '34) and fed as follows:

- Group A—30 days on diet containing toxic wheat lab. no. 459, then control diet.  
 Group B—20 days on diet containing toxic wheat lab. no. 459, then control diet.  
 Group C—10 days on diet containing toxic wheat lab. no. 459, then control diet.  
 Group D—Control diet entire period.

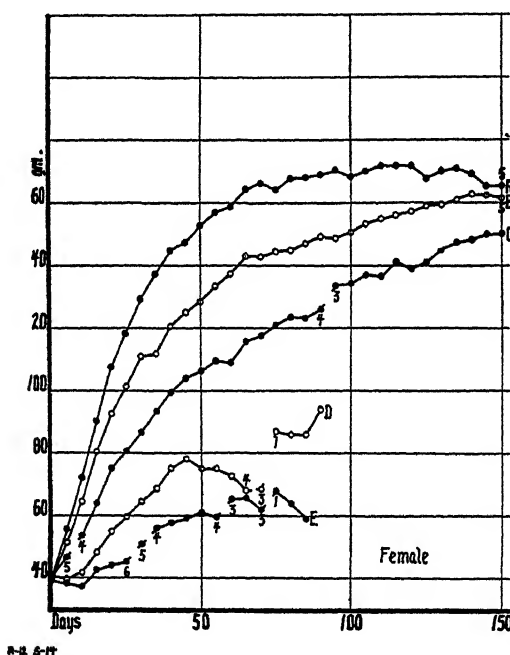


Fig. 2 Average growth curves of females (same as above).

The composition of the diet was the same as for the first series, except that wheat was used in place of corn. The growth curves and food intake curves of these rats are given in figure 3. In group A one rat died before the thirtieth day of experimentation when they were changed to the diet containing control wheat. Even though the others responded to the change of diets, seven had been affected so severely that they died by the one hundredth day of experimentation.

The other two were killed on the one hundred and seventy-fifth day.

In group B the same immediate response to change of diet is noted, but here again the damage to the system had been

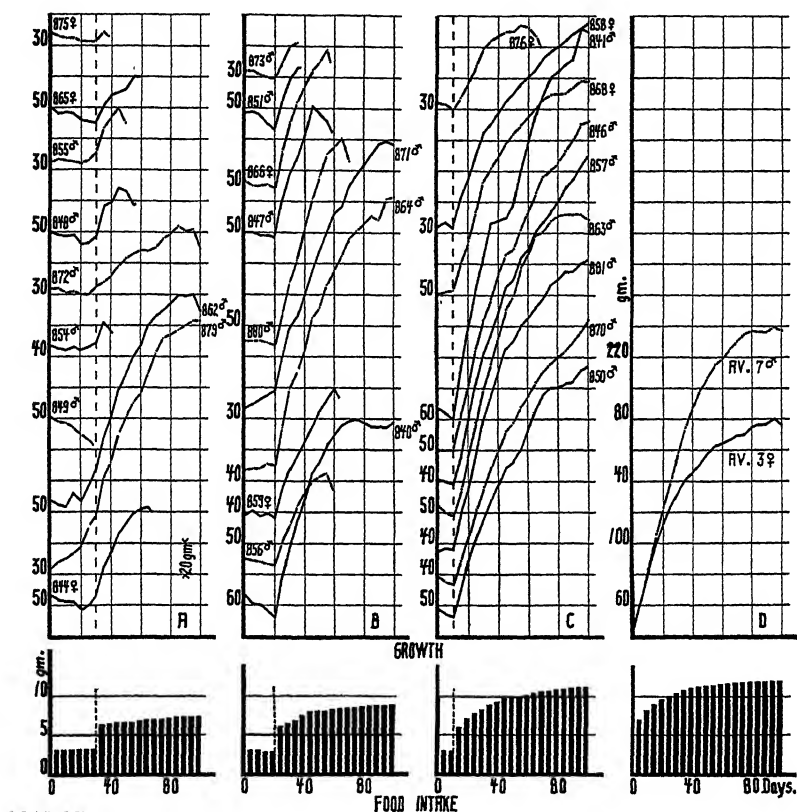


Fig. 3 Growth curves of rats fed: first 30 days on diet containing toxic wheat, then on diet containing control wheat; first 20 days on diet containing toxic wheat, then on diet containing control wheat; first 10 days on diet containing toxic wheat, then on diet containing control wheat. Control group. Food intake of rats obtaining toxic wheat first 30 days, first 20 days, first 10 days, and control.

so severe that it caused the death of seven out of ten rats by the seventieth day. In group C only one rat died (on sixty-eighth day). The others were killed on the one hundred and seventy-fifth day. The differences in food intakes in these

groups are very interesting. The sharp increases noted each time the rats are changed to the control diet are especially interesting.

It was of interest that four litter mates (one in each group) were living on the one hundred and seventy-fifth day. When these were killed, the weights of the liver, spleen, testes and epididymides were so different that they are given:

RAT NO.	GROUP	WEIGHT IN GRAMS						
		Body		Food consumed	Liver	Spleen	Testes	Epididymides
		Start	End					
862	A	57	226	1516	5.90 (2.61)	1.00 (0.44)	1.00 (0.44)	0.60 (0.27)
864	B	47	279	1858	7.35 (2.63)	0.80 (0.29)	1.10 (0.39)	0.65 (0.23)
863	C	54	284	1859	7.60 (2.68)	0.80 (0.28)	1.30 (0.46)	0.70 (0.25)
861	D	48	274	1941	9.00 (3.28)	0.55 (0.20)	3.05 (1.11)	1.15 (0.42)

Figures in parentheses are based on 100 gm. body weight.



Fig. A Testes, spleen and liver from male litter mates: Rat no. 861, control; 865, 10 days; 864, 20 days; 862, 30 days on diet containing toxic corn.

It will be noted that the body weights are fairly uniform. Therefore, the organ weights are not abnormal because of differences in growth, but are due to a pathological condition caused by the toxic foodstuff. The testes and epididymides were not as firm as those from control rats.

The gross appearance of the livers, spleens, and testes are shown in figure A. In the post mortems of the other rats similar conditions were noted, but in varying degrees.

#### DISCUSSION

From the first series in which the question of the effect of dilution of toxic foodstuffs was studied, the conclusion might be drawn that all livestock in the affected area is subnormal to a greater or lesser degree. It has been shown that the toxic constituent is not constant in the plant materials grown in any one area (Franke, '34); just as the soil varies in its chemical composition, and weather conditions vary, so also the composition of plants grown thereon may vary. The fact must not be overlooked that subnormal animals may exist without being noticed.

In the second series when the rats were fed diets containing toxic wheat for 30-, 20-, and 10-day periods followed by control diet, it must be recognized that the restriction of food intake was entirely voluntary. Stewart ('16), by underfeeding from 3 to 10 weeks, and following this by generous feeding, came to the conclusion that there was "a marked tendency of the body as a whole in young rats to recover its weight after a period of maintenance is likewise characteristic of the various organs and parts." Jackson and Stewart ('18) concluded that the suppression of growth in young rats affects their subsequent capacity to grow according to the age of the rats and the length of severity of the underfeeding. That is to say, the effect is inversely proportional to the age of the animal and directly proportional to the length of time underfed.

Jackson ('25) later states, "It is well known that recovery from inanition is generally possible if adequate nutrient is provided before the extreme stage is reached," but he calls attention to the fact that there is disagreement as to the possibility of a permanent stunting in the later growth of the body. He also takes up the question of the cause of death from inanition, either total or partial, and concludes that the

immediate cause may vary according to circumstances. Death may be a direct result of inanition or an indirect result caused by toxins in the circulation, or it may be due to a lowered resistance to infection known to be a result of various types of inanition, either total or partial. He states, "On the other hand, since most diseases, especially the chronic disorders, interfere more or less with the process of nutrition, inanition is usually present as a complication."

It is felt that this statement covers the work described in this series of papers in that the usual question of the lack of essential proteins, salts, vitamins and water probably does not enter into this work. However, many of the pathological symptoms generally connected with these can be observed.

When one considers that the animals do regain body weights by being fed control wheat, a concurrence with these statements of Stewart and Jackson will be found, but when the organs, especially the liver, spleen and kidneys, and in many cases the reproductive organs are studied, it is found that profound pathological changes have taken place so that the normal development of these organs probably does not take place.

#### CONCLUSIONS

A dilution of toxic grain so that only 17.5 per cent is contained in the diet showed definite depressed growth rates and also caused deaths.

Concentrations of 35 per cent and more caused still greater depressions of growth, and a greater number of deaths.

The effect of feeding diets containing toxic wheat for 30-, 20-, and 10-day periods, followed by control diet, was studied. Pathological effects resulted even for the 10-day period on toxic diet. Although normal growth was resumed when the rats were changed to the control diet, the damage to the organs was never repaired.

The pathological changes decreased as the intake of toxic food decreased.

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# A NEW TOXICANT OCCURRING NATURALLY IN CERTAIN SAMPLES OF PLANT FOODSTUFFS <sup>1</sup>

## XI. THE EFFECT OF FEEDING TOXIC AND CONTROL FOODSTUFFS ALTERNATELY

KURT W. FRANKE

*Department of Experiment Station Chemistry, South Dakota State College,  
Brookings*

FIVE FIGURES

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In a previous publication the author (Franke, '35) reported the effect of feeding rats diets containing toxic foodstuffs in varying amounts and for different time periods. In the following paper is reported the effect of feeding alternately diets containing toxic and control corn to simulate the consumption of vegetation by grazing animals.

### EXPERIMENTAL

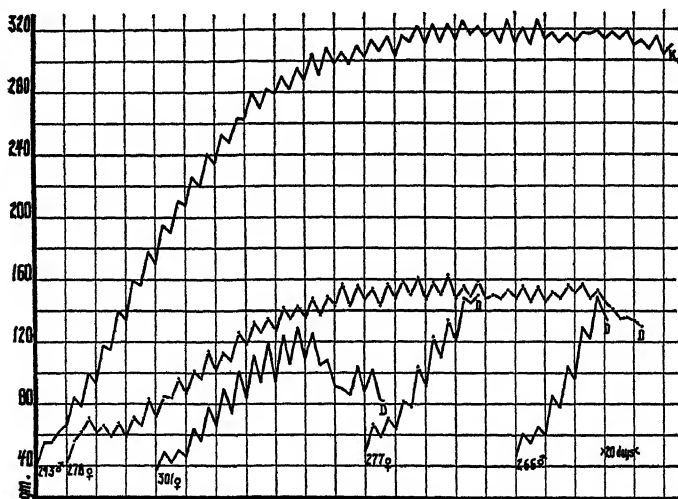
The rats used in this experiment were of Wistar Institute origin and had been weaned at 21 days of age and maintained for 1 week on a stock diet. Litter mates were then divided into four groups of five each. The rats were weighed every 5 days, and the food intake was weighed daily. Each rat was kept in an individual cage (Burr and Burr, '29).

<sup>1</sup>Published with the permission of the director of the South Dakota Agricultural Experiment Station as communication no. 18 from the Department of Experiment Station Chemistry. These investigations are being carried out under the Purnell Fund and with the cooperation of the Bureau of Chemistry and Soils, Bureau of Plant Industry, Bureau of Animal Industry, and Bureau of Home Economics of the United States Department of Agriculture.

The composition of the diets was as follows:

	<i>Per cent</i>
Ground whole corn	70.0
Casein	11.0
Sucrose	15.0
Lard	2.0
Calcium carbonate	1.4
Sodium chloride	0.6
Total	100.0

NOTE: One diet contained toxic corn laboratory no. 388, and the other contained control corn.



R-1, 5-15

Fig. 1 Growth curves of group A, fed alternately for 5-day periods, starting with diet containing control corn, then diet with toxic corn.

Four groups of rats were fed as follows:

- Group A Fed alternating 5-day periods Control and toxic diets
- Group B Fed alternating 5-day periods Toxic and control diets
- Group C Fed alternating 10-day periods Toxic and control diets
- Group D Fed alternating 15-day periods Toxic and control diets

The growth curves for group A are shown in figure 1. The rhythmic gain and loss of weight correlate with the food intake. The first death in this group occurred on the sixty-

second day of experimentation (rat no. 266 M) while the last rat (no. 293 M) was killed on the four hundred and twenty-fifth day.

In group B only one death had occurred on the three hundred and second day (no. 302 F). The others were killed on the four hundred and twenty-fifth day. The same rhythmic response in growth (fig. 2) and food consumption took place.

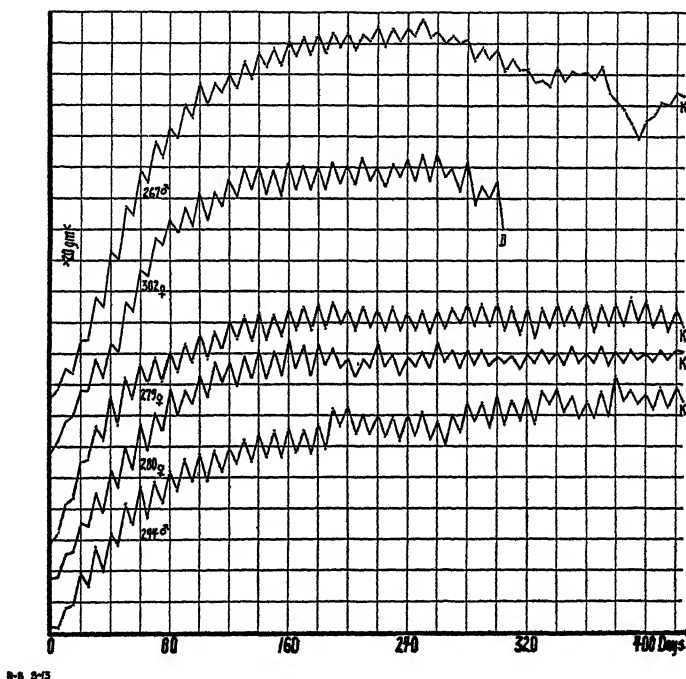
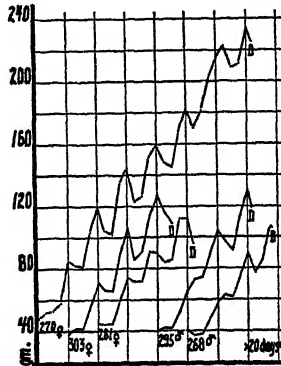


Fig. 2 Growth curves of group B. Fed alternately for 5-day periods, starting with diet containing toxic corn, then diet with control corn. Starting weights of the rats were: 294 M, 44 gm.; 280 F, 45 gm.; 279 F, 49 gm.; 302 F, 36 gm.; and 267 M, 42 gm.

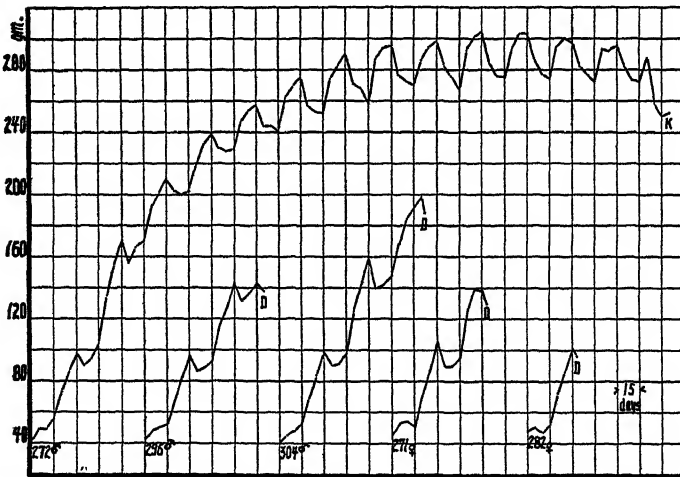
Since these rats were all litter mates, it is difficult to explain why only one rat should have died in group B while four died in group A. The growth of the rats in group C (10-day alternation) is shown in figure 3. Only one rat lived to the one hundred and forty-fourth day. This group showed the same rhythmic response in growth and food consumption as was observed in the other groups.

The shortest span of life was in the 15-day alternating group (group D) as shown by rat 282 F (32 days), while one survivor was killed on the four hundred and twenty-fifth day.



R-9 5-15

Fig. 3 Growth curves of group C. Fed alternately for 10-day periods, starting with diet containing affected corn, then diet with control corn.



R-10 5-15

Fig. 4 Growth curves of group D. Fed alternately for 15-day periods, starting with diet containing affected corn, then diet with control corn.

A very rhythmic food consumption for this rat is shown in figure 4. The next longest survivor lived only to the ninety-seventh day.

The external appearances and gross pathological conditions varied from practically normal to the severe conditions previously described (Franke, '34).

The food consumption for the males is shown in figure 5. The food consumption of the females varied in exactly the same manner, but averaged slightly lower.

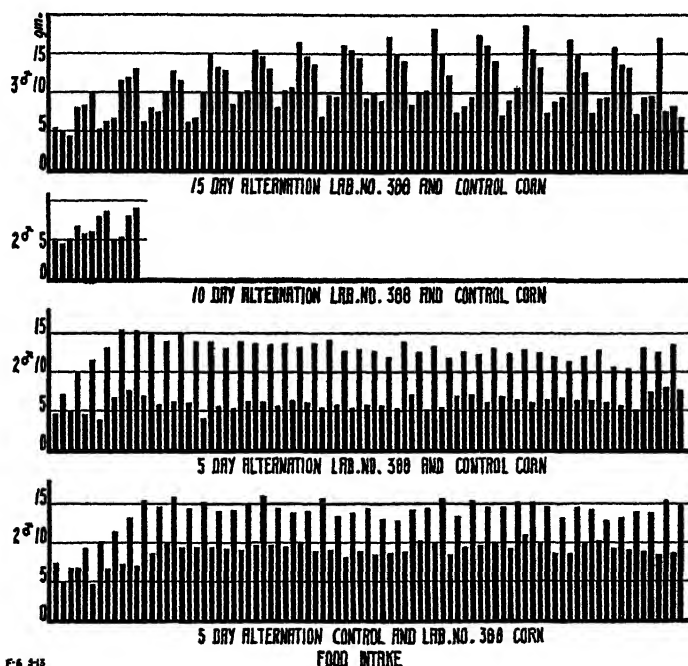


Fig. 5 Food intake of males in groups A, B, C and D, respectively.

#### DISCUSSION

In this series of alternating diets a rhythmic gain and loss of weight took place with a net gain on every change to control diet. This increased the body weight to a final weight approaching that normally obtained in controls on this diet. It is well to remember that an animal grazing might be in a position to alternate good with affected foods in shorter periods of time than those given in this series.

The only other reports in which similar rhythmic gain and loss of growth are shown are those in which forced periodic starvation occurred. Kopec and Latyszewski ('31 a, '31 b, '32 a, '32 b, '32 c) found that on an inadequate diet such as wheat, the control mice soon ceased to grow. The growth of the experimental group fed every other day was even lower. The growth rate of the experimental group on the days when fed was lower than the growth rate of the control rats on the same day, and resulted in a mean decrease in body weight. These results were obtained in two series. Both of these groups resumed growth when placed on an adequate diet, but the experimental groups in both series made the better growth. Kopec felt that the unusually rapid growth of the experimental group was due to a certain storage of growth capacity during the preceding period of intermittent starvation. Autopsies revealed that the unusual gain in weight of the experimental group was not due to abnormal accumulations of food or excrements in the alimentary tract.

In another series, during the first period of experimentation, he starved groups of mice 1 day out of 2, 1 day out of 3, 1 day out of 5, and 1 day out of 7, and designated them as ' $\frac{1}{2}$ ,' ' $\frac{1}{3}$ ,' ' $\frac{1}{5}$ ' and ' $\frac{1}{7}$ ,' respectively. By basing his conclusions on the growth during the first period he found that the negative changes in body weight on starvation days diminished in proportion to less intense starvation. Where animals were in individual cages the growth rate on fed days exceeded the control rate except in the case of the ' $\frac{1}{2}$ ' group. This was not the case when four animals were in a cage, probably explained in that the greater activity of the animals overbalanced such a factor as increased warmth.

Robertson, Marston and Walters ('34) reported that intermittent starvation 2 days in every 7 resulted in an immediate 12 per cent body weight loss, which was regained on access to food. The mean body weights of the starved animals became greater than those of the controls after both had been fed freely.

It appears, on comparing the growth of the surviving rats in the alternately fed groups with the controls of other series, as if a similar higher mean weight was obtained by alternate feeding. This is of such interest that further studies should be carried out, and if possible the factor which is definitely involved should be determined.

#### CONCLUSION

The alternate feeding of toxic diets and control diets gave growth and food consumption curves of rhythmic decreases and increases in all groups when the diets were changed. The intervals were of 5-, 10- and 15-day alternations.

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# THE IRON REQUIREMENT OF THE NORMAL HUMAN ADULT

GEORGE E. FARRAR, JR., AND S. MILTON GOLDHAMER

*Thomas Henry Simpson Memorial Institute for Medical Research,  
University of Michigan, Ann Arbor*

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Measurement of either the minimal or the optimal amount of iron necessary in the daily diet of the normal adult has seldom been attempted. Information concerning the normal daily iron requirement is urgently needed in the solution of problems arising in the recent widespread study of the anemias. The lack of extensive information concerning the quantitative metabolism of iron may well be explained by the difficulties encountered in the analysis of iron in biological materials, the small amount of iron metabolized by the human organism and the impossibility of obtaining a palatable iron-free or even a very low iron containing diet which is otherwise adequate for prolonged human consumption. At the University of Michigan Hospital normal young adults were eating accurately weighed diets during the course of insensible loss of weight experiments. These subjects have afforded a rare opportunity to observe the iron exchange in the human receiving a diet of low iron content.

## PREVIOUS OBSERVATIONS

An estimate of the daily iron requirement was first sought in the determination of the amount of iron excreted by the fasting human. In the classical study by Lehman and his associates (1893), the iron excreted in the stool of Cetti during a 10-day fast was 7.3 mg. per day, and of Breithaupt during a 6-day fast 8.0 mg. per day. Urinary iron was not

considered in this study and the nature of the analytical method employed for the iron determinations was not described. Since the period of fasting was very short, the iron of the food present in the gastro-intestinal tract at the beginning of the fast must account for an appreciable amount of the stool iron observed. Furthermore, the loss of body tissue during fasting could account for some excretory iron which would not be excreted under normal dietary conditions. Benedict's ('15) study of a man fasting for a month unfortunately did not include measurement of the iron excreted.

Stockman (1895) analyzed the diets served in two Edinburgh hospitals and nurses' homes, as well as duplicate diets to those ordinarily eaten by himself and his friends. The iron content of these diets varied between 6 and 11 mg. per day. The daily food intake of two chlorotic girls was analyzed on 2 successive days. These extremely limited and inadequate diets varied between 1.3 and 3.2 mg. of iron per day. Stockman's analytical method involved ashing the entire diet and titrating the reduced solution of the ash with potassium permanganate. His analyses of the iron content of some common foods are similar to those obtained by Peterson and Elvehjem ('28). Stockman and Greig (1897) reported iron balance studies for the last 3 days of 8-day diet periods, using normal adult subjects. One person receiving 6.2 mg. of iron per day excreted 6.3 mg. daily. A young woman ingesting 3.5 mg. per day excreted 3.7 mg. In two other experiments the excretion greatly exceeded the intake. Stockman's values for urinary iron are likely much too high.

Von Wendt ('05) made an extensive study of the protein and salt requirement of a normal adult which is of little significance for iron metabolism because of the almost daily changes made in the diet which do not allow time for the gastro-intestinal tract to empty itself of the previous days' higher or lower iron containing diet, and because of the use of Neumann's wet ash iodometric analytical method whose gross inaccuracy was demonstrated by Lintzel ('28) in a critical study. Von Wendt's data show an increase in stool iron for several days following a day with a high iron intake.

A more satisfactory study was carried out by Sherman ('07). During 3 days, an adult male ate a diet of crackers and milk containing 1690 calories, 63 gm. of protein and 5.7 mg. of iron daily. The average stool iron during these 3 days was 5.3 mg. per day plus an average urine iron of 0.2 mg., a total of 5.5 mg. excreted per day, a positive balance of 0.2 mg. per day. In the other two experiments the subjects developed diarrhea on the third day and the excretion of iron as might be expected exceeded the intake. Considering the absence of an adjustment period of at least 3 days on this cracker and milk diet before the iron excretion was measured and the difficulties inherent in the volumetric permanganate method of iron analysis, this subject was roughly in iron balance with a daily intake of 5.7 mg. of iron.

This problem was reinvestigated by Lintzel ('29). He first ('28) studied various analytical methods for iron determination and was concerned particularly with the iron content of normal urine. He developed an ingenious but laborious method for determining small amounts of iron and reported that normal adult male urine contained less than 0.01 mg. of iron daily. Lanyar, Lieb and Verdino ('33) confirmed this absence of iron in normal urine with Lintzel's method. Lintzel ('29) obtained similar negative results in urine with the volumetric titanium method and later ('33) with the bipyridyl method. The accuracy of Lintzel's first method has been confirmed by Farrar in this laboratory, but the urine of five normal subjects has varied between 0.02 and 0.03 mg. of iron per 100 cc. which is about twenty times the amount found by Lintzel, and corresponds to the values reported by Sherman ('07), by Rose and her associates ('30) and by Hanzal and Bing ('34). Lintzel fed a normal young male subject a constant diet containing 13.8 mg. per day for 4 days. During these 4 days the stool iron averaged 14 mg. daily. During the next 6 days the subject received a constant diet consisting of boiled rice, butter (especially prepared), egg white, tea and sugar in amounts giving a total daily iron intake of 0.9 mg. During the last 3 days of this diet period

the stool iron averaged 0.87 mg. per day. During the following 6 days the subject ate the first diet again, containing 13.8 mg. of iron a day. During the last 3 days of this period the stool iron averaged 13.8 mg. per day. The total iron intake of the 16-day period was 143.4 mg.; the total stool iron was 143.7 mg. Similar experiments were carried out with diets containing 1.6, 2.1 and 3.6 mg. of iron per day. The stool iron always corresponded closely with the food iron, requiring 2 to 3 days after a change in intake to reach equilibrium. Lintzel further found in these normal subjects that large doses of inorganic iron led to retention only on the first day of their administration. The problem of absorption and reexcretion of iron by the gastro-intestinal tract is not within the realm of a balance experiment.

Davidson and his associates ('33) studied the iron intake of the Aberdeen poor. Although some of the women and children were anemic, there was no correlation between the incidence of anemia and the iron content of the diet including diets affording only 6 mg. of iron daily over long periods of time. Sherman's ('07) dietary survey of various sections of the United States revealed a variation of daily iron intake from 7 mg. for the Alabama negro to 31 mg. in the Northern lumber camps.

The recent studies of Elvehjem, Hart and Sherman ('33) and of Sherman, Elvehjem and Hart ('34), concerning the availability of iron in many foods (the proportion of the total iron content of a food which the gastro-intestinal tract and the organism is able to utilize) has added a most important point of view in the study of iron metabolism. The total iron content of the diet is of less importance than the amount of iron actually available for the use of the body in the foods employed.<sup>1</sup> On this basis subject 'W' (described below) was

<sup>1</sup> The availability of the total iron content of some foods studied by simultaneous chemical (bipyridyl) and biological (milk anemia in young rats) methods in Elvehjem's laboratory is as follows: beef liver 70 per cent; beef heart 70 per cent; beef steak 50 per cent; wheat 50 per cent; oats 50 per cent; oysters 25 per cent; spinach 20 per cent. Hematin iron is not used by the gastro-intestinal tract.

receiving about 3 mg. of available iron a day when the total iron of the diet was 5 mg.

Several studies of the iron requirement of the infant and growing child have appeared, but this paper is not concerned with growth or abnormal aspects of iron metabolism.

#### EXPERIMENTAL

Except for short periods no satisfactory measurements of the iron requirement of the normal adult are available. At the University Hospital, Drs. L. H. Newburgh and F. H. Lashmet have been carrying out insensible loss of weight measurements on young adults under very carefully controlled conditions over periods of several months' duration. Because of their accurately measured, constant, simple diets, such subjects are ideal for a study of iron exchange. Precautions against iron contamination were instituted in the regime of four of these subjects. The details of this procedure which measures the total caloric exchange in such normal subjects living their usual active lives were reported by Newburgh, Wiley and Lashmet ('31). The iron content of the foods in use was determined. Toward the end of the period of observation the iron excreted in the urine and stool was measured and the usual routine blood examinations were performed, including blood iron determinations.

Incident to these observations an extensive study of the accuracy of analytical methods for iron determination in biological materials has been carried out by Farrar ('35). The thiocyanate analytical method described by Stugart ('31) as modified by Elvehjem ('34) was adopted in this laboratory. Analyses were performed in triplicate; iron was added to one of the three samples to test its recovery.<sup>2</sup>

A restricted number of simple foods were used in varying amounts. The constituents of the diet for each day were accurately weighed on a beam balance (sensitivity 0.1 gm.)

<sup>2</sup>Since the accuracy of the analytical method is  $\pm 0.1$  mg., the differences observed between the amounts of iron ingested and excreted are within the limits of error of the method.

individually, and then collectively as a check, by Doctor Newburgh or Doctor Lashmet, each morning. This ration was placed in a refrigerator and consumed by the subject in two or three portions. The milk and cream were obtained daily from the local dairy. Repeated analyses showed a surprising constancy of the iron content. The other foods were obtained in large lots. One subject (W) drank distilled water, the other three tap water, from individual thermos jugs which were weighed daily. This tap water contains an appreciable amount of iron which is included in the calculated iron intake. All of these subjects received a maintenance number of calories and were in nitrogen balance. Contamination of the excreta with iron was avoided by the use of new unchipped enamel ware.

Blood iron was determined by Hanzal's ('33) method. Hemoglobin was measured with the Sahli method using instruments standardized by the oxygen capacity method. Bureau of Standards instruments were used for the red and white blood cell counts. The menstrual hemoglobin loss was ascertained by the following rough method: Extract the napkins repeatedly with cold water in the ice box until the extractions are colorless, filter, treat an aliquot portion with dilute hydrochloric acid, compare in a colorimeter with an acid hematin solution of known concentration. Obviously such a method produces only a low approximation of the blood discharged.

Table 1 shows the iron content of the foods employed.

Tables 2, 3, 4 and 5 show for each subject the daily diet and the daily iron intake by periods as well as the stool, urine and blood iron where these were measured.

Table 6 summarizes the findings on all four cases.

Subject W, a male graduate student 26 years of age, lived on this regime for 316 days. For this entire period the average daily iron intake was 4.9 mg. During the last 31 days of this period the iron excretion was measured. The daily intake during this month averaged 5.2 mg., the stool iron averaged 5.2 mg. per day. In our experience normal male urine

has contained about 0.02 mg. of iron per 100 cc. using several analytical methods. At the end of 10 months on a diet containing a daily average of 4.9 mg. of iron this subject remained in iron balance. Furthermore, the blood iron, hemoglobin and red blood cell levels were within the normal range at the end of this period of low iron intake.

Subject V, a male graduate student 23 years of age, lived on this regime for 160 days. The average iron intake was 7.8 mg. per day. The urine and stool excreted for 5 days during the last month of this 5-month period were analyzed.

TABLE 1  
*Iron content of the food samples used in these diets*

FOOD	IRON
	<i>mg. per 100 gm. as fed</i>
Milk, whole, pasteurized	0.05
Cream, 40%	0.10
Cheese, Cheddar	0.80
Bread, white	0.22
Bread, whole wheat	1.75
Shredded wheat	4.00
Puffed wheat	4.10
Bran, washed	8.50
Butter	0.19
Jelly, grape	1.00
Apple sauce, canned	0.29
Grape fruit, canned	0.70
Tomato juice, canned	1.30
Ann Arbor tap water	0.08

The subject was in iron balance on an iron intake of 7.7 mg. per day. Like the first subject, the blood findings were normal.

Subject J was a patient 18 years of age with an arrested minimal pulmonary tuberculosis, who was continued on bed rest during this study. His diet averaged 7.1 mg. of iron per day for 4 months. The intake and excretion of iron were equal during 5 days of the last month of this study and there was no anemia at the beginning or the end of this 4-month period.



TABLE 2  
*Subject W daily diet*

DATES	2-29 to 3-24	3-25 to 5-4	5-5 to 6-29	6-30 to 7-14	7-15 to 8-31	9-1 to 9-11	9-12 to 10-13	10-14 to 10-27	10-28 to 12-3	12-4 to 12-12	12-13 to 12-29	12-30 to 1-12
Diet	2	3	4	5	6	7	8	9	10	11	12	13
Milk, whole	1900	1900	1900	1900	3032	2965	1500	1530	1500	1473	1610	1643
Cream, 40%	50	40	140	140	70	170	300	250	300	340	280	230
Bread, white	200	190	190	190	190	190	0	0	0	0	0	0
Bread, whole wheat	0	0	0	0	0	0	150	150	150	150	60	60
Butter	40	30	50	50	35	35	0	0	0	0	10	10
Jelly	0	0	0	0	0	0	96	100	96	80	0	0
Sugar	0	0	25	25	25	25	0	15	0	10	15	15
Shredded wheat	24	24	24	24	24	24	0	0	0	0	48	48
Grape fruit, canned	200	200	200	200	200	200	200	200	163	163	163	163
Bran, washed	0	5	5	5	5	5	0	0	0	0	0	0
Total iron intake per day	3.9	4.2	4.4	4.4	5.5	4.9	6.0	6.0	5.8	5.6	5.2	5.2
Fecal iron per day											5.4	5.0
Urine iron per day											0.2	0.2
Blood iron											56.3	

TABLE 3  
*Subject J daily diet*

DATES	10-11 to 11-24	11-25 to 12-4	12-5 to 12-14	12-15 to 12-21	12-22 to 12-23	12-24 to 1-8	1-9 to 1-18	1-19 to 1-28
Diet	A	B	C	D	E	F	G	H
Milk, whole	1000 (gm.)	1021	1053	1021	934	1000	1700	1700
Cream, 40 %	220 (gm.)	188	140	188	188	150	100	200
Bread, whole wheat	150 (gm.)	150	150	150	180	150	150	150
Butter	50 (gm.)	50	50	50	84	60	10	40
Shredded wheat	24 (gm.)	24	24	24	24	0	0	0
Grape fruit, canned	100 (gm.)	100	100	100	100	0	0	0
Jelly, grape	90 (gm.)	90	90	90	10	0	0	0
Sugar	25 (gm.)	14	13	14	14	10	10	10
Bran, washed	5 (gm.)	5	0	5	0	0	5	0
Puffed wheat	0 (gm.)	0	0	0	0	10	10	10
Tomato juice (canned)	0 (gm.)	0	0	0	0	100	100	100
Apple sauce, canned	0 (gm.)	0	0	0	0	150	100	100
Cheese, Cheddar	0 (gm.)	0	0	0	0	30	60	50
Tap water	1000 (gm.)	1000	1000	1000	1000	1000	1000	1000
Total iron intake per day	7.2 (mg.)	7.2	6.8	7.2	6.5	6.8	7.2	7.3
Stool iron per day								7.2
Urine iron per day								0.2
Blood iron								54.0



Subject R was a dietitian 24 years of age who lived on this regime for 41 days with an average iron intake of 9.1 mg. per day. She likewise was in iron balance and showed normal blood iron and hemoglobin levels. Her blood was examined

TABLE 5  
*Subject R daily diet*

DATES	1-8 to 1-24	1-25 to 2-17
Diet	A	B
Milk, whole (gm.)	1200	1400
Bread, whole wheat (gm.)	180	210
Shredded wheat (gm.)	48	48
Jelly (gm.)	65	85
Grape fruit, canned (gm.)	200	300
Sugar (gm.)	18	10
Tap water (gm.)	825	825
Total iron intake per day (mg.)	8.3	9.8
Stool iron per day (mg.)	8.0	
Urine iron per day (mg.)	0.2	
Blood iron (mg. %)		47.8

TABLE 6  
*Summary of four subjects*

SUBJECT	TOTAL NUMBER OF DAYS ON THE DIET	AVERAGE IRON INTAKE PER DAY	IRON BALANCE EXPERIMENT				BLOOD FINDINGS AT THE END OF THE WHOLE PERIOD	
			Days	Intake of iron per day	Output		Blood iron	Hemoglobin % (Sgall) 16.6 gm. = 100 %
					Stool iron average per day	Urine iron average per day		
W	316	mg.	31	mg.	mg.	mg.	mg.	98
V	160	7.8	5	7.7	7.6	0.2	52.8	94
J	110	7.1	5	7.3	7.2	0.2	54.0	97
R	41	9.1	17	8.3	8.0	0.2	47.8	80

tri-weekly throughout the 41 days. No significant changes occurred in the hemoglobin percentage and the red or white blood cell count. The amount of hemoglobin lost during a menstrual period which occurred during period 'B' of this

study was found to correspond to 33 cc. of the subject's blood or about 12.0 mg. of iron. A second dietitian, not included in this study, showed a similar hemoglobin loss. These figures correspond closely with those reported by Fowler and Barer ('35) for the amount of blood lost in a normal menstrual period. Because of the hemoglobin decomposition inherent in menstruation, as well as the decomposition occurring during the extraction process, and the other forms of iron present in the menstrual discharge (Ohlson and Daum, '35) our estimate of the amount of iron lost is certainly too low. Unfortunately the excreta during period 'B' were discarded before iron analyses were completed.

#### SUMMARY

1. A healthy male, 26 years of age, carried on his usual duties as a graduate student for 316 days on a diet whose average daily iron content was 4.9 mg. During the last 31 days of this period the subject was in iron balance when the diet contained 5.2 mg. of iron daily. The blood hemoglobin, blood iron and red blood cell levels were within normal limits.

2. Two other male subjects were likewise in iron balance after 4 and 5 months on diets containing 7.1 and 7.8 mg. of iron daily respectively, and their blood contained normal amounts of hemoglobin and red blood cells.

3. A young woman living for over a month on a diet containing 9.1 mg. of iron daily was in iron balance during the intermenstrual phase. The total menstrual blood loss represented 33 cc. of the subject's blood.

4. Normal urinary iron amounts to about 0.02 mg. per 100 cc. of urine.

5. These observations, together with those in the previous literature, indicate that the iron requirement of the normal adult male is not more than 5 mg. daily. The obvious importance of the availability of iron in the diet has only recently been demonstrated.

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# METABOLISM IN THE RAT OF THE NATURALLY OCCURRING ARSENIC OF SHRIMP AS COMPARED WITH ARSENIC TRIOXIDE

E. J. COULSON

*U. S. Bureau of Fisheries*<sup>1</sup>

AND

ROE E. REMINGTON AND KENNETH M. LYNCH

*Medical College of the State of South Carolina, Charleston*

ONE FIGURE

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Beginning with the announcement of Couerbe (1834) to the French academy a little over 100 years ago that he had found arsenic in the putrid bodies of human beings, a controversy has existed as to whether or not arsenic is a normal constituent of the animal organism. With the development of more sensitive and more accurate methods for the detection and determination of arsenic, there is a growing body of evidence that minute traces of arsenic are of wide-spread occurrence in normal plant and animal tissues.

Billeter and Marfurt ('23) have examined the organs and tissues of seventeen cadavers, ranging from newborn to 70 years of age (without arsenical medication) and found arsenic always present, increasing in amount with age. Fordyce, Rosen and Meyers ('22) found arsenic to be present in normal urine in about 75 per cent of forty cases, and in normal blood in 50 per cent of 130 cases, while Guthman and Grass ('32) found arsenic always present in the venous blood of women, averaging 0.64 part per million. A very interesting feature,

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according to Guthman and Grass is that during menstruation the arsenic concentration of the blood rises by 50 per cent to an average of 0.93 part per million. During pregnancy, the increase is still greater, reaching its maximum of 2.20 parts per million between the fifth and sixth months. In the later months of pregnancy, there is a gradual fall, but even at full term the value is still above the inter-menstrual value. Guthman and Grass suggest that this increase in blood arsenic may be related to growth processes and cell proliferation. Godon-nèche and Dastugue ('34) have also reported that the rate of growth and the date of metamorphosis of tadpoles are accelerated by solutions containing small amounts of arsenic.

The source of the arsenic in the urine, blood and organs of humans can readily be traced to the arsenic content of the dietary and water supply, particularly with the present day widespread use of arsenical sprays and of foods prepared by artificial processes. Nevertheless, Headden ('10) has reported the common occurrence of arsenic in virgin soils as well as in the plants growing thereon while the fact that sea foods contain arsenic is well known. It therefore seems evident that whether or not arsenic is of physiological importance to the body, it is always present.

Within the range of nutritive utilization, it has been assumed, without a great deal of experimental evidence, that mineral elements in the so-called 'natural' combination in which they occur in foods are better utilized than the inorganic salts. Evidence to the contrary, however, is accumulating. The iron of hematin, for example, is but poorly utilized (Elvehjem, '32) and that of meat and liver less readily than that of wheat (Rose, Vahlteich and MacLeod, '34), while for oysters, spinach, alfalfa and blood, only 25 per cent, or less, of the iron is available for hemoglobin building purposes (Sherman, Elvehjem and Hart, '34).

In the range of toxicity, arsenic trioxide, for example, is more toxic to higher animals than certain aromatic organic compounds of arsenic (the arsphenamines). It has also been shown that certain forms of copper as it occurs in foods and

the character of the diet which accompanies the feeding of inorganic copper salts may greatly influence the quantities of the metal which may be stored in the bodies of experimental animals (Coulson, Remington and Lynch, '34).

It is well known that practically all marine life is normally richer in arsenic than products from the land. Vertebrate sea fish from British and Swedish waters, according to Cox ('25), contain from 0.1 to 3.0 mg. arsenic (as  $\text{As}_2\text{O}_3$ ) per kilogram, fresh basis. Chapman ('26) analyzed a large number of shellfish and crustaceans from the coastal waters of the British Isles. His values varied from a minimum of 3 mg. per kilogram in the case of an oyster sample to a maximum of 174 mg. per kilogram in one sample of prawns and he found the sea water, from which these fish were taken, to contain arsenic in concentrations ranging from 0.14 to 1.0 part per million.

The arsenic content of some American shellfish and crustaceans as reported by White ('33) is lower than those reported by Chapman, but the values for vertebrate fish are somewhat higher than those reported by Cox. The results of these investigators agree, however, in that crustaceans contain more arsenic than any other sea animals.

In the course of our analyses of various sea foods, it was noted that shrimp grown in certain localities may contain relatively large quantities of arsenic. These shrimp seem to grow and thrive in spite of an arsenic content much higher than that of most other marine organisms. Table 1 shows the arsenic content of samples of shrimp which have been collected from various localities during the course of about a year and a half. The concentration of arsenic varies in these shrimp from 1.27 mg. per kilogram to 41.60. This variation is probably due to the character and arsenic content of the vegetation on the feeding grounds in the localities in which these shrimp grow.

An interesting feature of the results shown in table 1 is the variation in arsenic content of shrimp collected from the same locality. Samples collected near the vicinity of the

mouth of the Stono River near Charleston, S. C., on October 25 and 28, 1933, contained, respectively, 152 and 171 mg. per kilogram of arsenic (dry basis); while samples collected during May and June of the following year contained only 21 and 51 mg. per kilogram. Again in October, 1934, the arsenic content of a sample of shrimp collected from the same locality was 137 mg. per kilogram. This variation in arsenic content

TABLE 1  
*Arsenic content of shrimp (edible portion) from various localities*

DATE OF COLLECTION	SAMPLE NO.	FROM VICINITY OF	ARSENIC ( $\text{As}_2\text{O}_3$ ) CONTENT	
			Fresh basis	Dry basis
			<i>Milligrams per kilogram</i>	
June 15, 1933	102 <sup>1</sup>	Aransas Pass, Texas. Gulf shrimp	1.94	10.8
Sept. 25	116 <sup>1</sup>	Aransas Pass, Texas. Bay shrimp	2.44	13.0
Aug. 15	107 <sup>2</sup>	Savannah, Georgia	15.10	72.5
Aug. 15	108 <sup>2</sup>	Savannah, Georgia	9.10	28.8
Sept. 9	113 <sup>3</sup>	Mobile, Alabama	1.27	4.9
Oct. 13	117 <sup>1</sup>	Lake Salvador near Bayou Barataria, Louisiana	18.80	98.4
Sept. 12	114 <sup>2</sup>	Folly River near Charleston, S. C.	3.83	18.6
Sept. 26	115 <sup>2</sup>	Off Folly Beach, S. C. Deep sea shrimp	17.30	82.0
Oct. 25	120 <sup>2</sup>	Mouth of Stono River near Charleston, S. C.	36.60	152.0
Oct. 28	127 <sup>2</sup>	Mouth of Stono River near Charleston, S. C.	41.60	171.0
May 7, 1934	150 <sup>3</sup>	Mouth of Stono River near Charleston, S. C.	5.94	21.0
June 15	157 <sup>2</sup>	Mouth of Stono River near Charleston, S. C.	15.40	50.6
Oct. 23	198 <sup>2</sup>	Mouth of Stono River near Charleston, S. C.	30.70	137.0

<sup>1</sup> Canned shrimp.

<sup>2</sup> Peeled raw.

<sup>3</sup> Cooked and peeled.

of shrimp from the same locality suggests that there is some seasonal cause. Just what the significance of this may be or the source of the arsenic content of the shrimp from this particular locality is not known.

It is apparent that the availability of such a rich source of 'natural' or metabolized arsenic furnishes an excellent opportunity to study the relative metabolism, storage and toxicity of arsenic of these high arsenic containing foods as

against arsenic trioxide when fed to rats over a long period of time.

#### PLAN OF EXPERIMENT

Young rats of approximately 33 to 35 days of age were divided into five groups of sixteen animals each. The sexes were evenly divided in each group and were caged separately in large group cages with raised screen bottoms. Distilled water was given ad libitum.

The basic diet used was the Sherman diet 13 as modified by Russell, and has been described elsewhere (Levine, Remington and Culp, '31). In this investigation the usual proportions of the constituents were shifted slightly and had the following composition:

	<i>Per cent</i>
Ground whole wheat	59.3
Dried whole milk (Klim)	29.5
Meat and bone scraps	10.0
Sodium chloride	1.2
	<hr/> 100.0

In the shrimp diets the meat and bone scraps of the above described diet were replaced by dried shrimp. Two lots of dried shrimp were used; one lot (low arsenic shrimp) contained 10.4 mg. per kilogram of arsenic, and the other (high arsenic shrimp) contained 171 mg. per kilogram. The arsenic trioxide was added to the diets in the following manner: to 1 kg. of ground wheat in a large evaporating dish was added a solution consisting of 85 cc. of  $\text{As}_2\text{O}_3$  in water (1 cc. = 2 mg.  $\text{As}_2\text{O}_3$ ) to which was added 200 cc. of alcohol to facilitate mixing in the diet. This was mixed thoroughly and dried on a steam bath. After the addition of a sufficient amount of untreated ground wheat to bring the total weight up to the original 1 kg. (replacing the moisture which had been driven off), the wheat was reground and mixed thoroughly. The calculated amount of this arsenic wheat was then added to the basic diets, replacing an equivalent amount of untreated wheat.

The above described diets with their arsenic contents in milligrams per kilogram ( $\text{As}_2\text{O}_3$ ), as determined by the method of the Association of Official Agricultural Chemists ('30), are shown in table 2.

The diets were fed for a period of 12 months. At intervals of 3 months four representative animals from each group were killed and histological examinations made on the liver,

TABLE 2  
*Arsenic content ( $\text{As}_2\text{O}_3$ ) of arsenic fed rats*

	WEEKS	STOCK DIET	LOW ARSENIC SHRIMP DIET	HIGH ARSENIC SHRIMP DIET	STOCK DIET AND $\text{As}_2\text{O}_3$	LOW ARSENIC SHRIMP DIET AND $\text{As}_2\text{O}_3$
<i>Milligrams per kilogram</i>						
Arsenic content of diets		0.20	1.20	17.70	17.90	17.90
<i>Milligrams per rat</i>						
Total arsenic ingested	13	0.24	1.29	19.23	19.78	19.60
	24	0.46	2.49	36.26	36.32	36.27
	39	0.72	3.86	56.95	57.53	58.22
	52	0.95	5.42	75.53	76.11	75.98
Total arsenic stored	13	....	0.11	0.13	3.73	3.57
	24	0.067	0.18	0.26	3.58	4.25
	39	0.088	0.15	0.30	4.09	4.59
	52	0.178	0.25	0.29	3.99	4.46
	54 <sup>1</sup>	....	0.23	0.16	3.13	2.95
Percentage of intake stored by rats	13	....	8.5	0.7	18.9	18.2
	24	14.6	7.2	0.7	9.9	11.7
	39	12.2	3.9	0.5	7.1	7.9
	52	18.7	4.6	0.4	5.2	5.9

<sup>1</sup> Arsenic feeding was discontinued for 2 weeks.

spleen and kidney. The remaining portions of the livers of each group were then pooled and analyzed for arsenic. The female rats of each group after the removal of the alimentary canal were passed through a food chopper and also analyzed for arsenic.

Accurate records of food consumption were kept on all groups for the purpose of calculating the amount of arsenic consumed and to observe whether the incorporation of arsenic

in the diet affected appetite or utilization, such an effect having been claimed by Sollman ('21).

### RESULTS

The food intake and rate of growth of the rats in the various groups were practically the same. The growth curves for

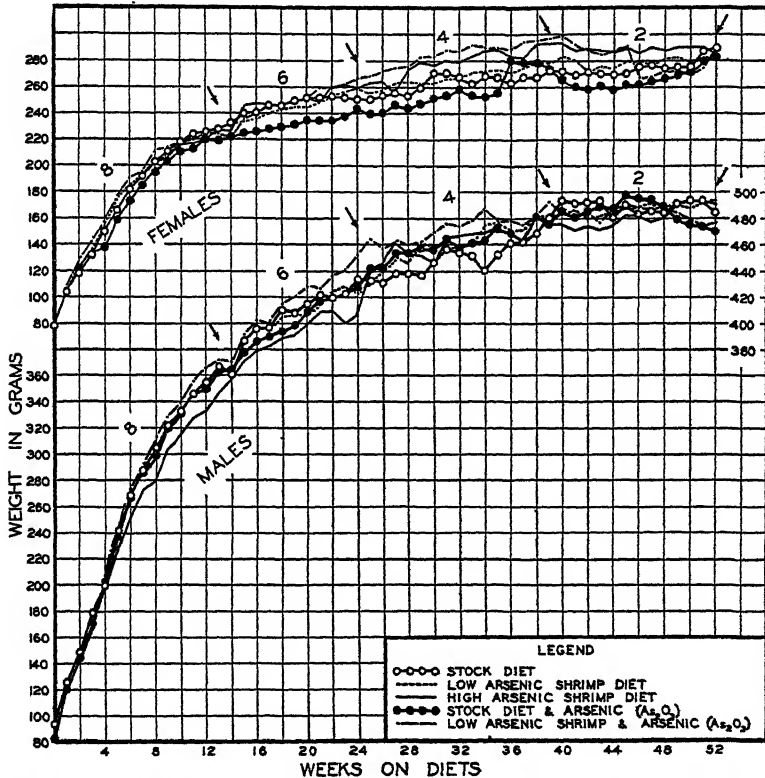


Fig.1 Showing growth rates of rats on diets of varying arsenic content derived from shrimp and from added arsenic trioxide. Arrows indicate point at which rats were killed for analysis and figures between arrows represent number of animals in each group during that portion of the curve.

the rats on the above described diets are shown in figure 1. For the purpose of conserving space, the food intake data are not shown; however, if desired, they may be calculated from the data given in table 2.

Table 2 shows the total amount of arsenic which was ingested by the rats as well as the total amount and percentage of the arsenic from the various diets which were stored by the rats between the time intervals at which animals were sacrificed for analysis. It can be seen from this table that the arsenic content of rats on the stock diet slowly but continually increased during the experimental period. This observation is in agreement with the previously mentioned work of Billeter and Marfurt ('23), who found that the arsenic content of humans increased with age. The carcasses of rats receiving the largest amount of arsenic in the form of shrimp (ninety times the amount in the control diet), contained from two to four times the quantity contained in controls. In marked contrast to this, the rats which had received approximately the same quantity of arsenic as arsenic trioxide contained from fifty-five to sixty-five times that in the control animals. The results also show that during the first 3 months of the feeding period only 0.7 per cent of the ingested 'shrimp arsenic' accumulated in the bodies of the rats, while more than 18 per cent of the inorganic arsenic trioxide was stored. After this period, the rats receiving the 'shrimp arsenic' continued to accumulate the element at about the same rate but apparently the rats receiving the inorganic arsenic trioxide had, some time within the first 3 months of the feeding period, reached an equilibrium in which no more storage of arsenic was taking place. It is, therefore, impossible to calculate from the above results the percentage of inorganic arsenic which was stored before this equilibrium had been reached. Undoubtedly the percentage stored would have been much higher had the first feeding period been of shorter duration.

In table 3 is shown the concentration of the arsenic in the livers of the animals on the various diets. Here again it can be seen that the animals receiving inorganic arsenic were able to store it in concentrations of nearly 100 times the 'normal' amounts, while the 'shrimp arsenic' was stored only to the extent of about seven times the 'normal' concentration. These results also show how arsenic accumulates in the liver in

greater concentrations than in the rest of the body. Thus livers of rats receiving the low arsenic-shrimp diet plus  $\text{As}_2\text{O}_3$  at the end of 13 weeks contained 57.7 mg. of arsenic per kilogram of fresh tissue, while the rest of the carcass contained only 14.9 mg. per kilogram. The liver, which consisted of only 5 per cent of the weight of the whole organism, contained 16 per cent of the arsenic.

After the rats had been on the arsenic diets for 1 year, arsenic feeding was discontinued for 2 weeks in order to obtain some idea of the rapidity with which metabolized arsenic is eliminated from the body. At the end of this time

TABLE 3  
*Arsenic content ( $\text{As}_2\text{O}_3$ ) of livers of arsenic fed rats*

WEEKS	STOCK DIET	LOW ARSENIC SHRIMP DIET	HIGH ARSENIC SHRIMP DIET	STOCK DIET AND $\text{As}_2\text{O}_3$	LOW ARSENIC SHRIMP DIET AND $\text{As}_2\text{O}_3$
<i>Milligrams per kilogram fresh tissue</i>					
13	0.43	1.71	3.13	52.30	57.70
24	0.72	2.48	3.41	60.10	71.40
39	0.83	2.20	3.94	56.50	61.20
52	0.92	2.21	2.82	49.10	56.60
54 <sup>1</sup>	....	2.10	2.47	38.30	41.80

<sup>1</sup> Arsenic feeding was discontinued for 2 weeks.

the remaining rats in each group were killed and analyzed for arsenic. The results shown in tables 2 and 3 indicate that about one-fourth to one-third of the arsenic is eliminated in this length of time.

There was no observable effect of the added arsenic on the physical vigor or appearance of any of the experimental animals. All of the rats appeared to be in healthy condition and, on autopsy, no gross abnormality of the viscera was observed. No histological evidence of injury to the spleen, liver or kidney could be detected when sections of these organs were compared with similar sections from the normal control animals in this series and in a previous large series of animals (Coulson, Remington and Lynch, '34).



## ARSENIC EXCRETION EXPERIMENTS

Excretion experiments were conducted on rats and on two human subjects in order to determine whether or not the arsenic compound of shrimp is absorbed from the gastrointestinal tract and to obtain some information upon the relative rate of excretion of 'shrimp arsenic' as compared to the inorganic form.

Four adult rats were selected from our stock colony and divided into two pairs. The animals were placed in cages designed to separate the urinary and fecal material, the arrangement consisting of a round galvanized iron wire cage suspended within a large glass funnel below the stem of which was suspended a glass ball in such a manner that the urine flowed around the ball and dropped through a small funnel into an Erlenmeyer flask. The feces, upon striking the glass ball, were deflected into a large beaker provided for that purpose. The urine and feces were collected daily. The funnel was washed each day with hot distilled water and the washings, containing the small amount of scattered food and some urine which had dried upon it, were added to the urinary material.

The urinary and fecal arsenic was determined daily for 1 week in order to establish a value for the normal daily excretion of the rats on the stock diet. At the end of this time, the rats were given the high arsenic containing diet for 1 day and then the stock diet until the excretion decreased to the normal value.

The high arsenic-shrimp diet contained 13.7 mg. per kilogram of arsenic in the form of the natural compound of shrimp and the stock-diet-plus-arsenic-trioxide was made up to contain the same concentration.

*Results of rat experiments*

The results of the excretion experiments are shown in table 4.

The average daily arsenic excretion of the rats on the stock diet was  $5.9 \gamma$  per day ( $\gamma \approx 0.001$  mg.) for group A and  $6.5 \gamma$

TABLE 4  
*Arsenic excretion in the rat*

GROUP A							GROUP B						
Time in days	Diet	Arsenic ingested	Arsenic eliminated			Arsenic recovered	Diet	Arsenic ingested	Arsenic eliminated			Arsenic recovered	
			Feces	Urine	Total				Feces	Urine	Total		
		$\gamma$	$\gamma$	$\gamma$	Per cent		$\gamma$	$\gamma$	$\gamma$	Per cent			
1	Stock	480	1.2	4.7	5.9	74.6	Stock	370	1.4	5.1	6.5	6.1	
2	Shrimp		14.1	350.0	364.1		As <sub>2</sub> O <sub>3</sub>		7.9	21.1	29.0		10.8
3	Stock		6.3	100.0	106.3		Stock		37.5	9.1	46.6		1.4
4	Stock		2.5	13.1	15.6		Stock		7.1	4.7	11.8		0.8
5	Stock		1.2	8.1	9.3		Stock		1.7	7.9	9.6		
6	Stock		1.7	4.4	6.1		Stock		1.7	4.7	6.4		
7	Stock		1.9	4.2	6.1		Stock		1.8	5.1	6.9		
	Stock	1.8	4.2	6.0		Stock	1.8	5.2	7.0				
Total						98.2	Total						19.1
8	As <sub>2</sub> O <sub>3</sub>	425	18.1	37.0	55.1	11.6	Shrimp	425	3.9	324.0	327.9	75.6	
9	Stock		26.2	6.3	32.5	6.3	Stock		3.9	85.0	88.9	19.4	
10	Stock		5.3	12.5	17.8	2.8	Stock		1.4	12.7	14.1	1.8	
11	Stock		1.6	5.5	7.1	0.3	Stock		1.4	8.1	9.5	0.7	
12	Stock		1.5	4.7	6.2		Stock		1.5	5.1	6.6		
Total						21.0	Total						97.5

for group B, about 80 per cent of the excreted arsenic being in the urine. The rats in group A were then given the high arsenic shrimp diet for 1 day and they consumed 35 gm. of the diet containing 480  $\gamma$  of arsenic. It can be seen from the table that during the 24 hours that they were consuming the diet 74.6 per cent of the ingested arsenic appeared in the excreta and 98.2 per cent was recovered within 4 days, at the end of which time the arsenic excretion had fallen to the normal level. On the other hand, the rats in group B which had consumed 27 gm. of the diet containing 370  $\gamma$  of inorganic arsenic had eliminated only 19.1 per cent of the ingested arsenic before the arsenic excretion had fallen to the normal level. In other words, 80 per cent of the ingested inorganic arsenic was retained in the bodies of the rats.

At the end of a week's time when it was certain that the arsenic excretion was proceeding at a normal rate, the experiment was repeated. This time group B received the shrimp diet and group A the inorganic arsenic. Again practically all of the ingested 'shrimp arsenic' was excreted in the first 4 days while 79 per cent of the inorganic arsenic was retained.

It is interesting to note that when the rats received arsenic in the form of shrimp about 96 per cent of the excreted arsenic appeared in the urine, but when inorganic arsenic was fed the excretion was about equally divided between the urine and feces. These results indicate that the arsenic compound of shrimp is very readily absorbed from the gastrointestinal tract but that it is rapidly and almost completely eliminated.

#### *Excretion experiments on human beings*

The results of rat experiments cannot always be applied to human beings, so in order to determine whether or not the excretion of arsenic is similar in these two subjects a similar experiment was conducted on two human subjects. No especial attempt was made to regulate the diet of the subjects during the course of the experiment with the exception that sea foods, which are known to contain arsenic, were avoided.

The result was that the normal arsenic excretion varied somewhat from day to day but it was felt that these variations would not be significant when compared to the relatively much larger amounts ingested and excreted when the shrimp sample was eaten.

TABLE 5  
*Arsenic excretion in human beings*

SUBJECT A						SUBJECT B					
Time in days	Arsenic ingested	Arsenic eliminated			Arsenic recovered	Arsenic ingested	Arsenic eliminated			Arsenic recovered	
		Feces	Urine	Total			Feces	Urine	Total		
	$\gamma$	$\gamma$	$\gamma$	$\gamma$	Per cent	$\gamma$	$\gamma$	$\gamma$	$\gamma$	Per cent	
1	Normal	7	26	33		Normal	15	29	43		
2	1180	23	1014	1037	84.9	980	18	780	798	77.0	
3	as	9	221	230	16.6		16	198	214	17.4	
4	as	6	149	155	10.3	as	11	90	101	5.9	
5	Shrimp	9	50	59	2.2	Shrimp	..	104	...	7.7	
6							..	78	...	5.0	
							..	44	...	1.5	
Total					114.0	Total					114.5
13	1000	19	308	327	29.4	1000	31	340	371	32.8	
14		..	200	....	17.4		..	258	...	22.9	
15		..	108	....	8.2		..	140	...	11.1	
16	as	..	102	....	7.6	as	..	150	...	12.1	
17	As <sub>2</sub> O <sub>3</sub>	..	79	....	5.3	As <sub>2</sub> O <sub>3</sub>	..	122	...	9.3	
18		..	69	....	4.3		..	98	...	6.9	
19		..	41	....	1.5		..	108	...	7.9	
Total					73.7	Total					103.0

*Results of excretion experiments on human beings*

The results of the above described experiments are shown in table 5.

Subject A ate 200 gm. of boiled shrimp containing 1180  $\gamma$  arsenic, while subject B ate 165 gm. containing 980  $\gamma$  arsenic. From the results shown in table 5, it can be seen that the elimination of ingested 'shrimp arsenic' proceeded at about the same rate in the human subjects as in the rat. Inorganic arsenic, however, although excreted more slowly than 'shrimp

arsenic' is apparently eliminated more completely in human beings than in the rat.

In a somewhat similar experiment reported by Chapman ('26), a person ate 1 pound of lobster containing 33 mg. arsenic and he recovered 24.3 mg. or 74 per cent of the ingested arsenic in the urine within 48 hours at the end of which time the experiment was terminated. Fecal elimination was not determined.

#### DISCUSSION

It seems probable that the arsenic in the quantities in which it occurs in shrimp does not exist in a simple inorganic form but must be bound up into a more or less complex organic combination as the result of the metabolic processes of the organism. Whether this is an attempt on the part of the organism to diminish the toxicity of the arsenic or whether a certain proportion of the arsenic is actually found favorable to its development is, of course, impossible to say.

The results reported here, however, show that there is a difference in the metabolism of arsenic as it occurs in shrimp as compared with inorganic arsenic when fed to rats. That the arsenic from the shrimp is absorbed from the gastrointestinal tract is shown by our observation that after the ingestion of shrimp practically all of the ingested arsenic may be recovered in the urine within a relatively short time, indicating that following the normal process of digestion, the arsenic containing substance in shrimp yields a soluble and readily diffusible product which is not decomposed in the body and is rapidly eliminated by the kidneys.

Further evidence of the complexity of the arsenic in shrimp is seen in the difficulties encountered in its estimation by the usual sulfuric and nitric acid digestion and subsequent Gutzeit determination (Remington, Coulson and von Kolnitz, '34). It was shown that even after the destruction of practically all of the organic matter by treatment with sulfuric and nitric acids only negligible traces of the arsenic present could be determined in the Gutzeit flask, nor could the arsenic be pre-

precipitated out by magnesia mixture as described by Gross ('33) for the elimination of the interference due to pyridine. However, if the sulfuric acid digest of the shrimp were subjected to a higher temperature after the addition of  $\text{CuSO}_4$  (as a catalyst), the complex arsenic compound was broken down and could be fully recovered in the Gutzeit determination.

The results of the experiments reported in this investigation are of interest not only for the light which they throw on the metabolism of arsenic but also as additional evidence that the manner in which inorganic elements are used in the body depends upon the source or form in which those elements are presented.

#### SUMMARY

1. Only a very small percentage of the arsenic from shrimp when fed to rats was stored in the animal organism, while inorganic arsenic when fed at the same level accumulated to the extent of fifty-five to sixty-five times the 'normal' concentration in the bodies of the animals and over 100 times the 'normal' concentration in the livers.

2. During the first 3 months of the feeding period 18 per cent of the ingested  $\text{As}_2\text{O}_3$  was stored in the bodies of the rats (as against 0.7 per cent for 'shrimp arsenic') and the total quantity stored within the first 3 months was not significantly increased by feeding the element for an additional 9 months.

3. Rats which received for a period of 12 months diets containing 17.9 mg. arsenic per kilogram in the form of shrimp and of added  $\text{As}_2\text{O}_3$  showed no evidence of toxicity in their growth, physical appearance and activity or by histological examination of the liver, spleen and kidney.

4. Following the normal process of digestion the arsenic containing substance in shrimp yields a soluble and readily diffusible product which is rapidly eliminated by the kidneys.

5. Arsenic occurs in shrimp in a complex combination and cannot be liberated in the animal organism.

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# THE NUTRITIVE VALUE OF ANIMAL TISSUES IN GROWTH, REPRODUCTION AND LACTATION <sup>1</sup>

## III. THE NUTRITIVE VALUE OF BEEF HEART, KIDNEY, ROUND AND LIVER AFTER HEATING AND AFTER ALCOHOL EXTRACTION

WALTER H. SEEGER AND H. A. MATTILL

*Biochemical Laboratory, State University of Iowa, Iowa City*

FIVE FIGURES

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In 1931 Smith began a series of feeding experiments with liver which were designed to segregate some of its nutritive components. As a first step in this segregation liver was subjected to prolonged extraction with hot alcohol until fat-free, and the residual protein and the extracted materials were examined for their nutritive value in growth and lactation. Such extracted liver fed in a balanced ration no longer supported growth and lactation as does the raw or dried whole tissue (Smith, '31), and relatively large amounts of yeast, cod liver oil or wheat germ oil failed to correct the deficiency (Smith and Seegers, '34). These observations, as well as those of Mapson ('33) pointed to the possibility of hitherto unrecognized accessories readily extractable from liver, whose absence was responsible for the poor nutritive value of rations containing alcohol-extracted liver. In the present work beef heart, round and kidney were similarly studied, and liver in greater detail. A study of the effect of heat on these animal tissues has also been made.

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## EXPERIMENTAL PROCEDURE

*Preparation of tissues.* The tissues were obtained fresh from the slaughter house, trimmed free of adhering fat, ground fine and dried in a Buflovak steam heated drier at 70 to 100°C. The steam pressure was kept below 1 pound per square inch and the drying of 100 pounds of fresh tissue was accomplished in about 4 hours. The tissues were therefore never heated above 100°C. and were subjected to that temperature for only a short time near the end of the drying operation. The products so obtained were the source material for all feeding experiments. The extractor used was of the continuous type briefly described by Sperry ('26).

*Animals.* In general only male rats were used because of their more rapid growth and uniformity of behavior, and their routine management has been described (Smith and Seegers, '34). Food consumption records were not obtained in all experiments; and only those which seem significant are presented.

*Rations.* In all the growth studies the protein was fed at a 15 per cent level ( $N \times 6.25$ ). In the nitrogen balance and digestibility studies the proteins of the four animal tissues were fed at a 5 per cent level. In figuring the composition of the rations the non-nitrogenous material of a tissue preparation was arbitrarily considered as carbohydrate. The general composition of all rations used is given in table 1.

## GROWTH STUDIES

To determine whether alcohol removed important nutrients from other animal tissues as it does from liver, a comparative study was made with kidney, heart, round and liver extracted with 95 per cent alcohol for 60 hours, and dried in a warm air current. Control animals were fed the respective dried tissues. The composite growth curves are given in figure 1. There was little difference in the growth rate on the diets containing the dried whole tissues. This is in line with the earlier work of McCollum, Simmonds and Parsons ('21) and of Hoagland and Snider ('26). The extracted meats in all

cases gave less satisfactory growth, kidney gave the most rapid, liver the least. It was not surprising to obtain slower growth on the extracted tissues. Rather more striking was the fact that in contrast to kidney, heart and round, the extraction of liver readily removed or destroyed so much of its nutritional value.

TABLE 1  
*Composition of rations*

I. Used in growth studies<sup>1</sup>

a) Alcohol extracted tissues<sup>2</sup>

	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Liver	18.7			
Round <sup>3</sup>		16.1		
Heart			16.5	
Kidney				16.3
Hydrogenated cottonseed oil <sup>4</sup>	15.0	15.0	15.0	15.0
Corn starch	59.8	62.4	62.0	62.2

b) Dried whole tissues

	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Liver	23.2			
Round <sup>3</sup>		20.2		
Heart			21.0	
Kidney				21.8
Hydrogenated cottonseed oil <sup>4</sup>	10.7	11.2	10.6	9.7
Corn starch	59.6	62.1	61.9	62.0

II. Used in nitrogen balance and digestibility studies<sup>5</sup>

	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Liver (dried whole)	7.4			
Extracted liver (130 hours) <sup>6</sup>		6.1		
Heated liver <sup>3</sup>			7.2	
Lard	15.0	15.0	15.0	15.0
Agar agar	2.0	2.0	2.0	2.0
Salt mixture	4.5	4.5	4.5	4.5
Corn starch	71.1	73.5	71.3	78.5
Nitrogen content	0.85	0.84	0.86	0.047

<sup>1</sup> All these rations contained, in addition, agar, 2 per cent; salt mixture (Hawk and Oser, '31), 4.5 per cent, and were supplemented daily with 0.4 cc. cod liver oil and 0.5 gm. yeast (kindly supplied by Northwestern Yeast Company).

<sup>2</sup> The extent of the extraction is stated in the text; 95 per cent alcohol was used.

<sup>3</sup> Included the retail cuts designated as hind shank, round and rump.

<sup>4</sup> Crisco.

<sup>5</sup> Rations containing round, heart, and kidney, were similarly set up to contain 5 per cent of protein.

<sup>6</sup> Supplemented daily with a vitamin B concentrate containing 5.2 mg. N, and with 4 drops of cod liver oil.

A separate comparative study of the nutritive value of beef and pork liver was also made. The raw tissues were dried as described, and the liver ration was modified (table 1) by including 4 per cent yeast and 2 per cent cod liver oil at the expense of an equivalent amount of starch. Fourteen male rats fed beef liver made an average gain of  $231 \pm 3.3$  gm. while thirteen male rats on pork liver gained  $223 \pm 3.2$  gm. during the same 81-day growth period. This slight difference,

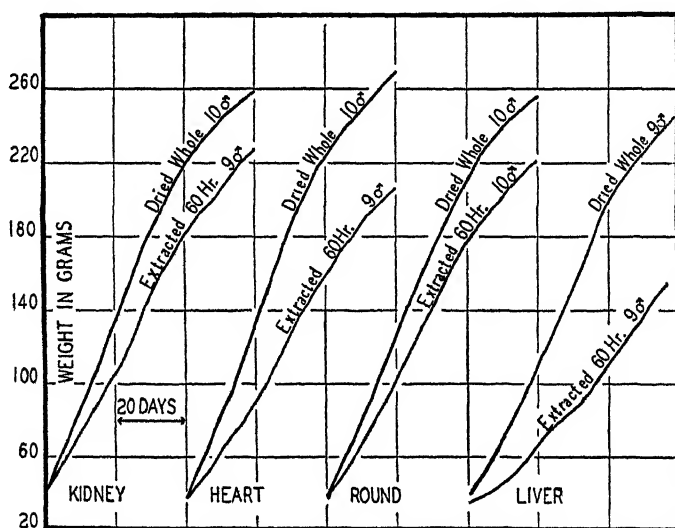


Fig. 1 Showing the composite growth curves of rats fed alcohol-extracted kidney, heart, round and liver. The curves for the respective dried whole tissues are also shown.

in view of the probable errors, hardly demonstrates beef liver to be superior to pork liver for growth purposes. Johnson and Palmer ('34), in a study which had primarily another purpose, fed the raw tissues as small supplements to an otherwise adequate diet and found them to increase growth to an equal extent. From food consumption records the efficiency quotients (Palmer and Kennedy, '31) were computed on the basis of total dry matter consumed (Morris, Palmer and Kennedy, '33). Both liver rations appeared to be equally well utilized. Figure 2 gives the efficiency quotient values for

different segments of the growth curve. It can be seen that during the period of rapid growth different values were obtained for the several segments of the growth curve without change of ration. The quotient tended to increase with advancing age, presumably largely because of increased maintenance costs. This consideration may not justify the application of an efficiency quotient determined on a young animal as a reference for that same animal in subsequent periods of growth. The efficiency index, which requires a standard procedure, appears to be more reliable but is less flexible in its use.

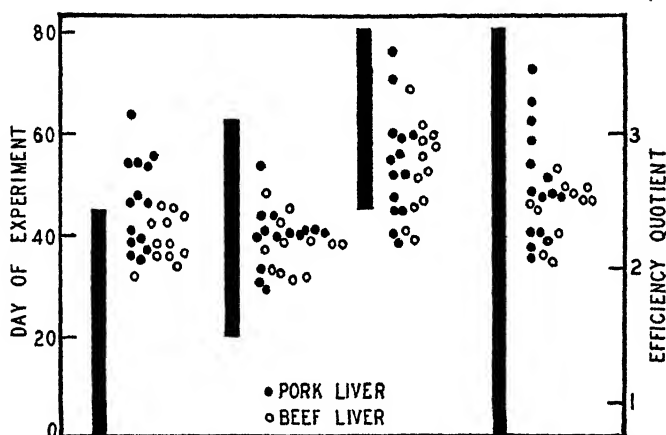


Fig. 2 Illustrating the efficiency quotient values for different segments of the growth curve. The bars represent the segment of the growth curve. The rats weighed from 40 to 50 gm. at the beginning of the experiment.

A preliminary experiment with alcohol-extracted tissues, conducted somewhat differently from the one described above, first emphasized the incipient deleterious effect of moderate high temperatures on nutritive value. After the routine extraction procedure the tissues were dried from 3 to 4 days in an electric oven at 90 to 100°C. This was for the purpose of removing volatile decomposition products which, as judged by odor, largely disappear during the first 24 hours. The rats used were slow growing, being of the same strain and group as those used by Berg ('34) who also considered them

so. Nevertheless the poor showing made by these animals (table 2) seems of real significance. The small amount of fresh round fed to one group increased the growth rate considerably.

These observations suggested that heating for different lengths of time even at temperatures as low as 100°C. might alter the nutritive value. The very slow growth of the animals fed treated liver, as compared with their growth on the other tissues similarly treated, indicated that the nutritive qualities of these meats are not uniformly retained after alcohol extraction and heating. In further studies (fig. 3) the heating

TABLE 2

*Average gains made in 50 days on 60-hour alcohol-extracted tissues subsequently dried at 100°C. for 3 to 4 days, and fed at a 15 per cent protein level*

<i>Ration</i>	<i>Male</i>	<i>Female</i>
Liver	70 (7) <sup>1</sup>	39 (6)
Heart	109 (6)	79 (4)
Round	102 (5)	90 (5)
Round + 0.5 gm. raw daily	139 (5)	
Kidney	111 (4)	80 (7)

<sup>1</sup> Number of animals; they weighed from 30 to 40 gm. at the beginning of the experiment.

and extraction times were varied. Liver extracted 1½ hours<sup>2</sup> was definitely less valuable for growth than dried whole liver. Extracted 60 hours it suffered greatly and large amounts of vitamin G, held by some workers to be especially abundant in liver, did not supply the deficiency. Heating such extracted material for a week at 100°C. further reduced its nutritive value, and another week of heating at that temperature had an additional detrimental effect.

Kidney, which gave the best growth of any of the 60-hour alcohol-extracted tissues, was extracted for 130 hours and heated 2 weeks at 100°C. After this treatment it nevertheless still promoted growth better than liver which had been extracted and heated for only half that time. Unless unknown

<sup>2</sup> This particular extraction procedure was similar to that used by Booher ('33) in preparing vitamin G concentrate from skim milk powder, and it was also used in preparing the vitamin G concentrate used in the present experiments.

accessories are concerned, this difference can be ascribed to differences in the properties of the proteins in these tissues. Since liver is more readily altered it was studied in greater detail.

An attempt was first made to segregate the effects of heat and alcohol. For this purpose the following preparations were fed: a) dried whole liver, b) the same liver heated in an electric oven for 2 weeks at 100°C., c) 130-hour alcohol-extracted liver, and d) liver refluxed with 95 per cent alcohol for 130 hours. The refluxed material was prepared by placing

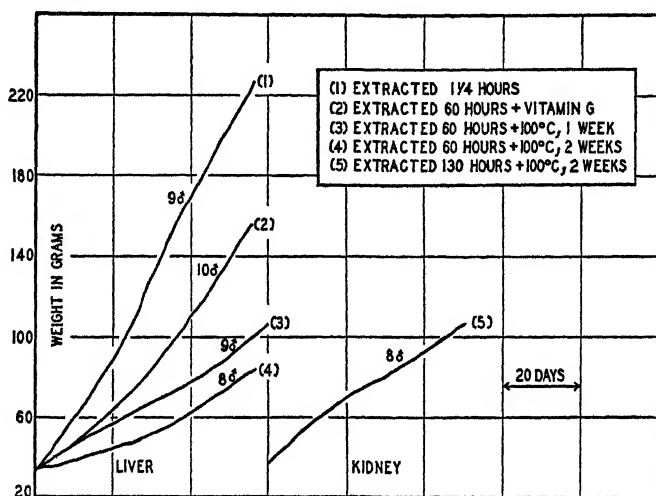


Fig. 3 Shows the effect of varying the hot alcohol extraction time, and the result of heating the residue at 100°C. for 1 and 2 weeks. The greater resistance of kidney is shown.

liver in a large balloon flask kept in a water bath at a temperature slightly above the boiling point of alcohol. After 130 hours the solvent was removed by vacuum distillation and the residue dried in a current of warm air. Exceedingly disagreeable odors (amines?) were given off during the refluxing and these were somewhat noticeable even after the material was incorporated in the basal ration.

The results are summarized in figure 4. Because of the variable behavior of the animals fed 130-hour extracted-liver

some typical individual growth curves are given. A number of deductions can be made from these curves. Heat alone even as low as  $100^{\circ}\text{C}$ ., decreased the nutritive value considerably. Boiling alcohol is much more harmful than dry heat at  $100^{\circ}\text{C}$ . showing that hot alcohol acts as a destructive agent in itself, independent of its solvent action. The very poor showing made by the 130-hour extracted material indicates that alcohol may also remove valuable nutrients. While this aspect has been studied further the results are difficult to interpret and are not discussed here. The removal of an

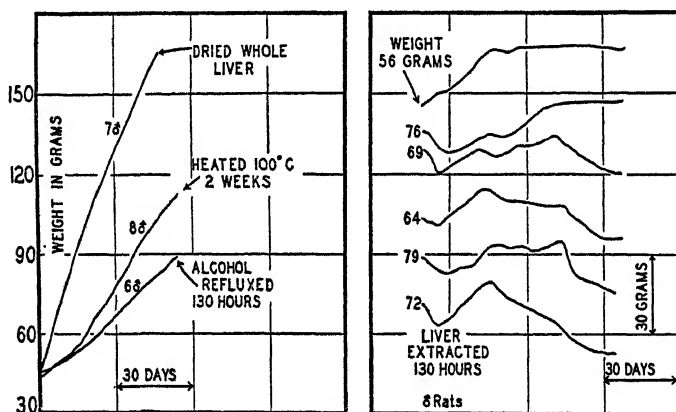


Fig. 4 Shows growth curves of controls and rats fed heated, alcohol-refluxed, and alcohol-extracted liver at a 15 per cent protein level.

unknown factor or factors was indeed suggested by Smith and Seegers ('34) as the primary alteration produced by alcohol extraction, but further information in that direction cannot be obtained until it is certain that the protein component remains unchanged by alcohol extraction.

If protein deficiency is the explanation for the differences between extracted kidney and liver it would follow that their proteins are so organized as to be differently affected by heat and alcohol. Windus, Catherwood and Rose ('31) obtained satisfactory growth on mixtures of highly purified amino acids supplemented with their unknown amino acid, yeast and cod liver oil. Animals on 130-hour alcohol-extracted liver

(15 per cent protein) supplemented with as large or larger amounts of yeast and cod liver oil are in a moribund condition in about 80 days. This comparison suggests that the primary cause of this nutritive failure is in a protein insufficiency. On the assumption that certain amino acids especially lysine and histidine (Greaves and Morgan, '34 a) were made unavailable as the result of alteration in the protein, attempts were made to improve a defective liver ration with ample amounts of lysine and histidine hydrochlorides (0.2 per cent each) but without success. A study of the nitrogen metabolism of animals on these liver diets was next undertaken.

#### BIOLOGICAL VALUE STUDIES

Biological value studies were conducted according to the method of Mitchell ('24). To insure comparable results it was planned to give each animal the same quantity of food in each period, and the same quantity of nitrogen in the protein feeding periods. This ideal was almost achieved, but difficulties were encountered during the last nitrogen-free period when some of the animals refused to consume all of their portions. In view of the uniformity secured, no correction was made in the computations for metabolic fecal nitrogen as related to food intake. Metabolic urinary nitrogen was based on body weight. Four-day preliminary periods were followed by a 6-day collection period, in one case 5 days.

The products studied were fed in the following order: nitrogen-free, 130-hour extracted liver, dried whole liver, liver heated at 100°C. for 2 weeks, and a final nitrogen-free period. Each animal received daily a yeast concentrate containing 5.2 mg. of nitrogen. In the calculation of the results (table 3) only the second nitrogen-free period was used, because a constant level of nitrogen excretion was just being reached at the end of the first nitrogen-free period, as indicated by the 2-day analyses made. This is a matter of considerable importance (French, '34). The use of the nitrogen-free data from the first period would have given higher and probably less trustworthy figures.



TABLE 3

*Average daily metabolic data showing biological value and digestibility of liver preparations*

RAT NO.	INITIAL WEIGHT	FINAL WEIGHT	FOOD INTAKE	N INTAKE	FECAL N	ABSORBED N	URINARY N	FOOD N UTILIZED	BIOLOGICAL VALUE	TRUE DIGESTIBILITY <sup>1</sup>
Nitrogen-free diet										
	gm.	gm.	gm.	mg.	mg.	mg.	mg.	mg.	per cent	per cent
1	196	190	9.00	5	10	5	38			
2	212	210	8.00	5	12	5	47			
3	212	203	9.00	5	11	5	46			
4	240	223	6.00	5	9	5	53			
5	196	189	8.00	5	12	5	49			
6	202	195	8.00	5	11	5	37			
7	232	220	7.00	5	9	5	56			
8	238	220	8.00	5	10	5	48			
Alcohol-extracted liver (130-hour) diet										
1	184	178	6.97	64	48	23	40	5	22	30
2	202	200	8.00	72	44	35	47	12	29	45
3	198	193	9.00	81	66	23	49	5	22	24
4	214	204	6.00	55	40	23	58	6	..	34
5	185	183	8.00	72	54	26	49	1	..	30
6	191	187	6.25	58	47	18	37	4	22	25
7	214	210	7.00	64	47	25	50	0	..	34
8	212	204	8.00	72	45	37	52	12	28	46
Average										34 ± 1.8
Dried whole liver diet										
1	176	176	7.00	64	17	54	47	28	52	83
2	204	205	7.98	73	17	63	49	39	62	85
3	192	194	8.98	81	16	73	57	47	60	90
4	200	200	5.98	56	13	50	61	19	38	88
5	181	190	7.98	73	14	66	45	45	68	90
6	184	182	6.00	56	12	51	42	31	61	90
7	210	213	7.98	73	16	65	49	41	63	88
8	202	202	7.98	73	15	67	57	36	54	91
Average										57 ± 2.1 88 ± 0.6
Heated liver (100°C., 2 weeks) diet <sup>2</sup>										
1	173	175	6.86	64	22	49	52	19	35	75
2	206	209	7.84	73	22	58	50	33	56	78
3	194	195	8.82	81	26	63	61	38	52	76
4	194	196	5.88	56	18	45	53	21	47	78
5	188	188	5.85	55	16	46	39	31	67	82
6	178	180	5.88	56	18	45	44	22	51	78
7	214	216	7.84	73	18	63	53	36	56	85
8	200	206	7.84	73	23	59	61	24	41	79
Average										51 ± 2.2 79 ± 0.6
Nitrogen-free diet										
1	170	158	5.00	5	7	5	20			
2	201	184	4.95	5	7	5	24			
3	188	176	6.76	5	8	5	29			
4	188	182	6.00	5	7	5	27			
5	180	168	5.00	5	7	5	23			
6	172	166	6.00	5	7	5	21			
7	210	205	7.13	5	8	5	24			
8	196	189	8.00	5	9	5	25			

<sup>1</sup> True digestibility =  $\frac{\text{Liver N intake} - \text{Liver N in feces}}{\text{Liver N intake}} \times 100$ .<sup>2</sup> Five-day collection period.

The biological value of 130-hour alcohol-extracted liver is strikingly low, and similarly the coefficient of digestibility is also low. Apparently after alcohol extraction only a part of the protein is digested yielding a poor assortment of amino acids of relatively small value for metabolic purposes.

The heated material was less strikingly altered, but apparently an analogous change took place. The biological value was only slightly lowered, and hardly enough, in view of the probable error, to account for the pronounced decline in the growth rate of rats fed such heated material (fig. 4). Again, as also with the extracted liver, the digestibility was lowered, but apparently only in proportion to the lowered biological value.

#### DIGESTIBILITY IN VITRO

In view of the marked changes in digestibility in the animal it seemed desirable to know whether they could be demonstrated by in vitro experiments. Comparative studies on the in vitro digestibility of the other animal tissues after heat and alcohol treatment would also indicate what changes, if any, might be expected in animal experiments.

*Procedure.* The amount of nitrogen contained in 3 gm. of alcohol-extracted liver (396 mg.) was arbitrarily chosen as the quantity of substrate. The other tissues were adjusted to this on the basis of their nitrogen content. No allowance was made for soluble nitrogen for the reason that the tissues were incorporated in rations on the basis of gross nitrogen content. To the substrate in a 200-cc. beaker was added 50 cc. of a 0.3 per cent  $\text{Na}_2\text{CO}_3$  solution containing 0.1 per cent NaF, and 10 cc. of a 3 per cent trypsin solution. This was kept at 37°C. for 24 hours. A control, without the enzyme solution, was included. At the end of 24 hours 10 cc. of the enzyme preparation were added to the control and 50 cc. of tungstic acid reagent (Folin-Wu) were added to all the samples which were then centrifuged and filtered. The clear filtrates were diluted to 200 cc. and duplicate Kjeldahl analyses and Sørensen titrations were made on aliquots.

As seen in table 4 the heated meats were less rapidly digested, especially round and liver, than the unheated. Similarly alcohol-extracted liver was not well digested while the same treatment of heart, round and kidney reduced the digestibility only slightly as measured by formol titration and even increased it as measured by the nitrogen in tungstic acid filtrates. The meaning of these contradictory figures has not been studied further. These more or less exploratory experiments on digestion in vitro therefore show marked reductions in digestibility of all the heated meats as compared with the unheated preparation, whereas alcohol extraction has an adverse effect on liver only.

TABLE 4  
*Digestibility of tissues in vitro, 37°C., 24 hours<sup>1</sup>*

TISSUE TREATMENT	FORMOL TITRATION CUBIC CENTIMETERS 0.1 N NaOH				PER CENT NITROGEN NOT PRECIPITATED BY TUNGSTIC ACID			
	Kidney	Heart	Round	Liver	Kidney	Heart	Round	Liver
Dried whole	50.8	45.0	50.0	29.5	46.2	48.5	57.5	31.8
Heated at 120°C., 2 weeks	22.6	10.9	6.7	10.7	35.6	26.5	18.9	12.1
Extracted 130 hours	42.8	40.4	40.0	10.7	74.3	66.6	60.6	15.9

<sup>1</sup> The figures are based on 132 mg. of substrate nitrogen.

#### DIGESTIBILITY EXPERIMENTS WITH RATS

The effect of heat on proteins especially at higher temperatures has been studied by Morgan ('31), Maynard, Bender and McCay ('32), Maynard and Tunison ('32), Fixsen and Jackson ('32), Schneider ('32), Greaves and Morgan ('34 a, '34 b), Wilgus, Norris and Heuser ('35), and many others. These workers all show that heat, if it is sufficiently intense, impairs the nutritive value of protein.

In view of these many observations and since in the in vitro experiments heat so consistently lowered the digestibility of all the meats it seemed important to verify this by animal

studies particularly with a view to determining whether for a given animal tissue there is a critical temperature beyond which it must be heated before an alteration is produced, and how rapidly alterations take place above that temperature.

These digestibility experiments were conducted in much the same way as described by Adolph and Wu ('34). The thirty-five rats used varied in weight from 60 to 150 gm. The plan was to feed the same amount of the ration daily, for 9 days and collect the excreta during the last 5 days of the period, but with the poorly digestible proteins the animals repeatedly refused their food allotment.<sup>3</sup> To insure approximate figures for true digestibility a protein-free period was also included and no correction was made for the influence of variable food intake on metabolic fecal nitrogen. The following formula was used in computing the digestibility:

$$\frac{N \text{ intake} - (\text{fecal N} - N \text{ fecal on N-free diet}) \times 100}{N \text{ intake}}$$

The general composition of the rations is given in table 1; the heating period was uniformly 3 days and the hot alcohol extraction 130 hours. The results are summarized in figure 5. The digestibility of all these meat proteins was impaired if the heating was sufficiently intense. Kidney was the most resistant and liver the least, while heart and round were between. Even at 110°C. there was some change in liver, but properly fried liver was well digested, presumably because the time of heating is short. Round was still well digested after heating at 120°C. and kidney after 130°C. The effects of heating seem to appear gradually and perhaps they play no important role in the domestic preparation of meats; when the time of heating is short the temperature must be much higher to produce a given effect (compare fig. 3). This

<sup>3</sup> The behavior of these animals was interesting; for example, when round heated at 120°C. for 3 days was fed, they consumed all of their food, but when they were shifted to round heated at 130°C. they promptly refused some of it. In the case of kidney this refusal first occurred when the tissue had been heated at 140°C. The taste of the food, the appetite and tissue requirements doubtless all contributed to this response.

is in general agreement with the conclusions of Scheunert and Bischoff ('30). In contrast to the lowered digestibility of heated meats, that of alcohol extracted tissues was not altered from the normal except in the case of liver (compare table 3).

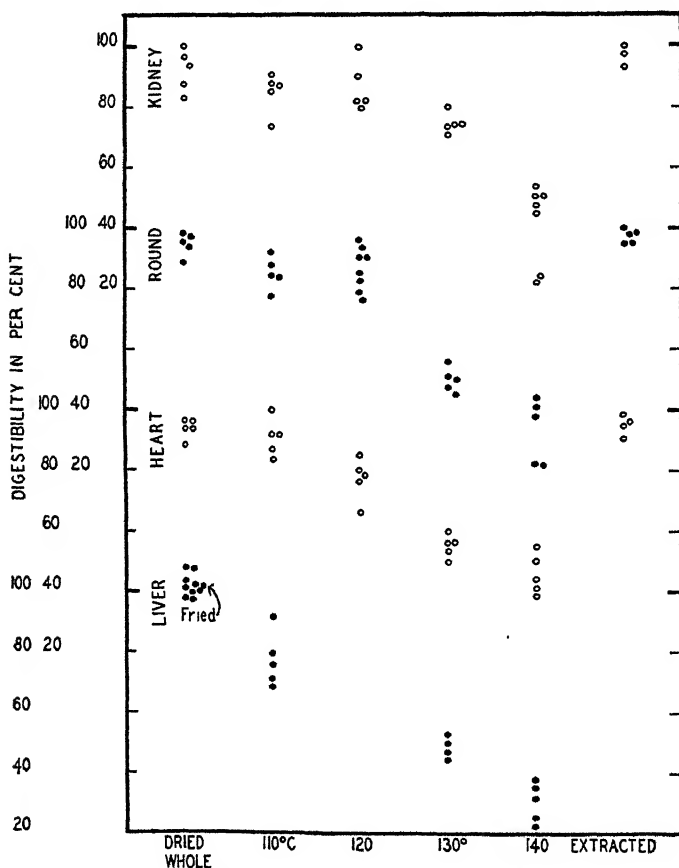


Fig. 5 Shows the digestibility of meats heated at different temperatures for 3 days, or extracted with hot alcohol for 130 hours.

The figures obtained in the in vitro digestion experiments are in rather striking agreement with those obtained on animals.

#### DISCUSSION

Although it is difficult to distinguish between the effects of heat and alcohol, this series of experiments seems to indi-

cate that alcohol extraction lessens the nutritive value of animal tissues primarily because it removes important dietary accessories, while heating, 1) inactivates such of these as are present in the tissue, and 2) if sufficiently intense or prolonged, modifies the character of the tissue protein in the direction of lowered digestibility.

Recently Wilgus, Morris and Heuser ('35) centered a nutrition investigation of haddock meal on the theory that the lowered growth promoting properties, after heating, are chiefly due to a change in the proteins and also to the destruction of some of the growth promoting activity of the vitamin G complex. While our own data do not in any way indicate which accessories are destroyed, they point to a thermal destruction of growth promoting substances at temperatures where the proteins are not altered. The poor growth on liver heated at 100°C. for 2 weeks (fig. 4) can hardly have been due to the slightly altered protein fed at a 15 per cent level, when the digestibility and biological value of that protein are so little affected (table 3). Had the temperature been slightly higher (110 to 120°C.) such a distinction could not have been made because the digestibility of liver is considerably reduced by such heating. There are no growth data on the other meats after heating. It has been shown, however, that liver proteins are more readily altered by heat than those of other meats, and it is reasonable to assume that their accessories would also be destroyed by heat at temperatures below those required to alter their proteins. When heat is used to destroy accessories in liver tissue (110 to 120°C.) the protein is also made inferior for growth, because it is made indigestible. Prolonged alcohol extraction accomplishes a similar change in liver proteins but not in those of other meats.

Disregarding the vitamin components of a meat, there is limited information which points to yet another cumulative effect, concerned with the alteration of the protein by alcohol extraction and subsequent heating. Thus when kidney was extracted for 130 hours and heated for 2 weeks at 100°C., the biological value was 50 per cent and the digestibility 77 per

cent (corrected), as shown by a limited number of experiments. If alcohol extracted kidney is so completely digestible (fig. 5) and if kidney itself must be heated above 130°C. before its digestibility is lowered one would hardly predict such low figures for alcohol extracted and subsequently heated kidney, especially when kidney is the most resistant to heat. The protein may conceivably be more susceptible to heat alteration after alcohol extraction (compare also table 2). The lowered biological value and digestibility of the alcohol extracted and heated kidney contrasted with the relatively small effect of alcohol alone or heat alone suggests such a possibility. The slow growth rate on this kidney preparation (fig. 3) is thus due to the removal or the destruction by heat (or both) of such accessories as were not adequately supplied by yeast and cod liver oil supplements; and to the lowered nutritive value of the protein.

The inferiority of liver tissue after alcohol-extracting or heating, implies that the protein of liver is peculiarly sensitive to alteration by heat and by hot alcohol, while the other meats are relatively unaffected and are apparently as well utilized after alcohol extraction as before. Their decreased nutritive value after extraction only, may be due solely to the loss of accessories (fig. 1), while liver suffers a double disadvantage in the loss of accessories and in the alteration of the protein by hot alcohol.

The nature of the chemical change induced by heat in animal proteins, especially liver, and by hot alcohol extraction of liver, is suggestive of racemization in that the proteins become refractory to the action of digestive ferments *in vitro* and *in vivo*.

#### SUMMARY

There was little difference in the growth rate of rats fed beef heart, kidney, round and liver at a 15 per cent level of protein in an otherwise apparently complete ration; pork liver was practically equal to beef liver in its capacity to promote growth. When these tissues were extracted in a continuous

extractor for 60 hours with hot 95 per cent alcohol growth was less rapid and was especially slow on extracted liver. Except with liver, whose proteins are altered by such extraction, the difference between the extracted and the unextracted meats can be ascribed solely to the removal of nutrients not adequately supplied by the yeast and cod liver oil supplements.

When the ration contained 130-hour alcohol-extracted liver in place of the 60-hour extracted it did not support growth and the animals died. In contrast to similarly extracted round, heart, and kidney such extracted liver is only slightly digestible and has a low biological value.

Although liver heated at 100°C. for 2 weeks no longer possessed its original capacity to support rapid growth in a balanced ration, its digestibility and the biological value were not lowered appreciably. Above 100°C. there was progressive lowering of the digestibility of the protein as the prolonged heating (3 days) became more intense. Heart and round were resistant to change at 120°C., while kidney was still digestible after heating at 130°C. At higher temperatures these meats also became refractory to digestive enzymes in the animal body as well as in vitro. These changes in digestibility probably play no role in the domestic cooking of meats, but are of importance in the preparation of pure proteins for nutrition studies.

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# SOME EFFECTS OF COD LIVER OIL AND WHEAT GERM ON THE RETENTION OF IRON, NITROGEN, PHOSPHORUS, CALCIUM AND MAGNESIUM DURING HUMAN PREGNANCY<sup>1</sup>

CALLIE MAE COONS AND R. R. COONS

*Department of Agricultural Chemistry Research, Oklahoma Agricultural and Mechanical College, Stillwater*

ONE FIGURE

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The use of continuous controlled experiments to measure the effects of specific dietary factors on the retention of minerals during human pregnancy has been seldom employed. Numerous difficulties concur to hinder these studies from being carried out over long periods of time with the subject restricted to a constant diet and routine of living habits. The time has come, however, in our stage of information when such procedures can be applied safely and ethically with corresponding gains in the type and quality of data obtained.

The results reported in this paper covered 101 days of continuous regulated diet during the last half of pregnancy. During 82 of the days the diet was weighed, while 19 days represented an intermission for possible recovery from therapy during which period the diet remained almost the same but no weighings or collections were made. Analyses are presented for eighteen metabolic periods of 4 days each during which balances of nitrogen, iron, phosphorus, calcium and magnesium were determined and records of creatinine

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elimination, urine volume, pH and specific gravity obtained.

Two questions which, for reasons to be discussed below, seemed eminently in need of investigation under these controlled experimental conditions concerned the quantitative effect of cod liver oil on calcium and phosphorus metabolism and the influence of some part of the vitamin B complex on nitrogen and iron retention during pregnancy. An effort was made to study the two simultaneously.

#### LITERATURE

In most of the studies in metabolism during human pregnancy reported in the literature the diet has been self-chosen and more or less variable from day to day. One case described by Macy and co-workers ('31) received a specified diet for 21 days divided into two non-continuous periods. Examples of prolonged studies with subjects receiving the varied home diets are those of Wilson ('16), two cases for 19 and 15 weeks each, and Hoffström's one case for 23 weeks ('10).

The benefits of cod liver oil on mineral metabolism in adults has not been demonstrated as conclusively as with children, despite its widespread use in pregnancy. Improvements noted usually have been slight. The women of Toverud's group ('31) who received cod liver oil showed some increases in retention of calcium but responded more favorably to higher mineral intakes. Two of the subjects previously reported by the writer (Coons and Blunt, '30; Coons et al., '34) were receiving cod liver oil routinely but showed no marked advantages over others of the groups in the retention of calcium and phosphorus. For clinical purposes, therefore, it has seemed worthwhile to study the effect on mineral retention during gestation when cod liver oil was added to a diet reasonably adequate in calcium and phosphorus. Furthermore, in the light of the decidedly superior retention of these two elements shown by women receiving reasonable amounts of potent sunshine (Coons et al., '35), it was desirable to measure the response to cod liver oil therapy under

more controlled conditions to know to what extent it could substitute for sunlight in its effect on calcium and phosphorus metabolism during gestation.

Interest in the factors affecting iron and nitrogen retention has been indicated in previous reports from this laboratory. Iron balances with a group of Chicago women showed fair retentions as compared to fetal needs (Coons, '32), while those with a group of Oklahoma women receiving even better iron intakes indicated poor utilization of iron simultaneous with excellent retentions of calcium (Coons et al., '35). Concentrates of vitamin B have shown a supplementary effect on the diets of college women of this section (Coons et al., '31). Since whole grain cereals were lacking in both sets of diets (Coons, '33), and since individual exceptions in the pregnancy group showed best iron retention, it was suggested that the deficiency might be some component of, or a factor associated with, the vitamin B complex. Reputed values of whole grain cereals in hemoglobin regeneration supported this view (Rose and Vahlteich, '32). Harris ('34) commenting recently on various reports of the relationship of vitamin B to anemia has referred to "the effect of a vitamin B deficiency in bringing about some error in iron metabolism of a nature not yet clearly understood."

Some factor associated with vitamin G and responsible for remission in certain anemias is being widely investigated at present. Varying degrees of success have been reported for marmite (Wills, '33), rice polishings (Miller and Rhoads, '34), yeast and wheat embryo (Ungley and James, '34), when used as substitutes for liver therapy in various anemias. Reports of studies concerning the effect of any vitamin B preparation on the utilization of dietary iron during a period of normally increased iron demand seem, therefore, timely at least.

#### EXPERIMENTAL STUDIES

*The subject.* The primipara used for this study was experienced as a subject for constant diet regimen as well as balance experiment procedures. Prior to the beginning of

pregnancy she had served 28 days on self-chosen diets with collections of excreta and at another time 36 days in a similar experiment requiring a restricted constant diet as well as collections (Reder and Coons).

The pregravid studies had shown that the subject could remain in slight negative balance on calcium intakes of 0.30 to 0.35 gm. daily and fluctuated from positive to negative on intakes of 1.0 gm. or over. Weight records scattered over the previous 8 years showed less than 5 pounds maximum variation from the 142 which existed at the beginning of this pregnancy and which was only 2 pounds below the average for her height and age. Also there had been no loss of time from work due to illness over a period of 10 years prior to this experiment. These facts indicated a fairly stable condition of health and reasonably adequate dietary habits and therefore a subject who could be regarded as 'normal' and with average nutritional reserves for the metabolism studies which were planned.

The pregnancy was uneventful except for a slight rise in blood pressure which developed during the last 2 weeks antepartum and which reached a maximum of 148/80 mm., but this was 4 weeks after the termination of the metabolism work. Gestation lasted 293 days, 42 weeks, after the beginning of the last menstrual period. The infant weighed 9 pounds, 9 ounces.

Metabolic studies including control and preliminary periods extended from the twenty-first to the thirty-sixth weeks, covering the seasons from mid-January to the last of April. In order to measure the possible effect of cod liver oil it was necessary that the subject avoid sunshine throughout all control and supplemented periods. This was accomplished by work indoors during daylight hours and in a north room most of the time. Similar precautions for protection from light were taken when conveyance was necessary in daytime. All activities were reduced to a regular daily schedule.

*The diet.* The diet planned was one as well suited to the taste preferences of the subject as experimental conditions

would allow. Meat was omitted from the first, lest later threatening signs of toxemia should necessitate radical changes in the basic diet or termination of the experiment entirely. Weight gains during the first half of the study justified a reduction in the amounts of certain high calorie foods for the last half. The diet was planned to contain about 1.2 gm. of calcium daily in addition to fair amounts of other minerals because this quantity of calcium seemed nearly adequate for women living under the influence of the sunlight of this region.

For the first half of the study, series A, the basic diet consisted daily of the following items with quantities stated in grams.

Breakfast: orange juice, strained, 200; grape-nuts cereal, 50; milk, 300.

Lunch: Iceberg lettuce, 100; mayonnaise, 20; butter, 50; whole wheat bread, 150; tomato juice, canned, 200; peaches, canned halves, 200; graham crackers, 50; eggs, 100.

Supper: milk, 400; potatoes, boiled in jackets and peeled, 200; peas, canned, 100; peaches, canned halves, 200; graham crackers, 50.

Distilled water, ad libitum. No salt in cooking or serving.

The items were identical for the second half, series B, but with reductions in certain quantities:

Whole wheat bread, reduced from 150 to 100 gm. daily

Butter, from 50 to 30 gm.

Potatoes, from 200 to 100 gm.

Eggs, from 100 to 50 gm.

A wheat germ preparation,<sup>2</sup> 30 gm. daily, or the ash of 30 gm., supplemented the above diet in certain periods and in others 15 gm. of standard cod liver oil<sup>3</sup> were supplied daily. The ash of the wheat embryo preparation was used alone in one period of each series because it was impossible to find a concentrate which did not change appreciably the mineral content of the diet and thereby prevent definite conclusions as to the supplementary effect from the vitamins alone.

The source, variety and brand of all foods used remained the same throughout both series. Eggs came from the same

<sup>2</sup> Squibb's Vitavose.

<sup>3</sup> Mead's Standardized Cod Liver Oil, purchased in open market.

flock of pure bred hens, bread from the same bakery and the same oven output each day, and potatoes were bought in two lots, one for each series. Canned foods, such as peaches, peas and tomato juice were purchased in case lots, but each can was not opened until needed for use, and the contents were drained of liquids before being weighed. The quality of lettuce varied greatly because of the market season covered by the experiment. The same may have been true for eggs, since the experiment included both early and late laying periods. Doubtless the composition of the bread varied much from time to time, as did also the iron content of foods from cans, particularly tomato juice. In spite of strict precautions the composition of the analyzed diets showed considerable variation and emphasizes the necessity for actual analysis of each diet as the only accurate procedure for balance experiments.

*Collections.* Each experimental series consisted of nine 4-day collection periods preceded by a 4-day preliminary period and followed by a 1-day lag period, throughout which the weighed diet remained the same. The methods of collections and analysis were identical with those regularly employed in this laboratory (Coons, '30; Coons et al., '35). An aliquot of each item of the diet was weighed into a composite which was analyzed for each element. This procedure seemed more practical and accurate for a prolonged series than did the preparation and preservation of each dietary item in homogeneous mixtures and quantities to last throughout the study. Supplements, when used, were added to the composite for analysis in the same aliquots as were other ingredients.

Urine was not analyzed for iron as for all other elements because previous studies had demonstrated that the amount excreted by way of urine was too small to affect the final balance appreciably and that the urinary excretion of iron was surprisingly constant from period to period for each individual (Coons, '32; Coons et al., '35).

## RESULTS AND DISCUSSION

The intake and outgo balances for iron, nitrogen, phosphorus, calcium and magnesium are shown in tables 1 and 2, while the graph in figure 1 demonstrates the interrelation between the curves of storage for these elements.

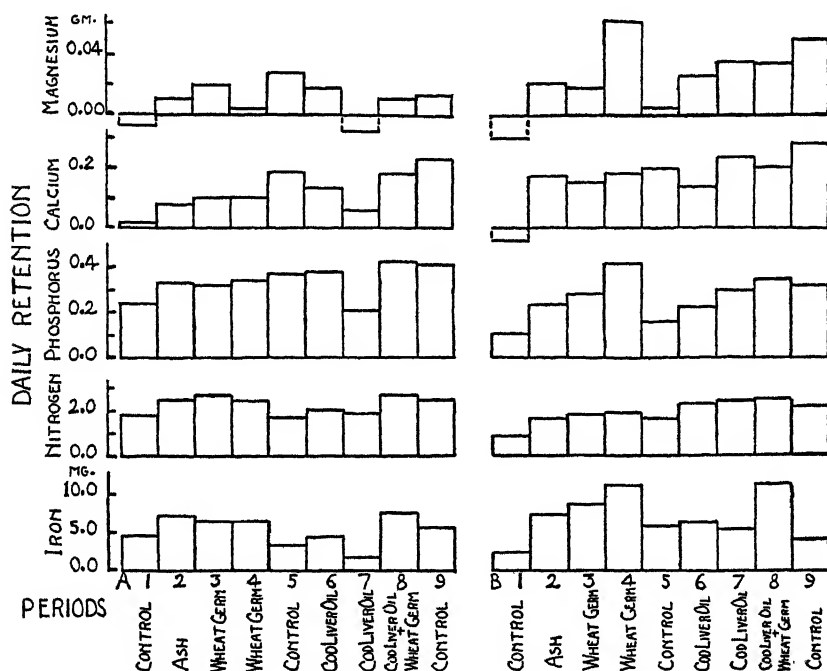


Fig. 1 The effect of various supplements to the basic diet on the rates of retention of iron, nitrogen, phosphorus, calcium and magnesium during the last half of pregnancy.

*Iron.* Both intake and storage of iron were good. The basic diet supplied approximately 18 to 20 mg. of iron daily, with 6 to 8 gm. added through the wheat germ preparation in four periods of each series. In all periods the intake was much above the 15 mg. recommended for adult dietaries (Sherman, '32) and approximated the 20 mg. suggested as tentative standards for pregnancy (Macy and Hunscher, '34).



TABLE 1  
*The effect of cod liver oil and wheat germ on the retention of iron, nitrogen and phosphorus during pregnancy*  
 (daily averages)

SERIES AND PERIOD	SUPPLEMENT TO BASIC DIET	WEEK 1 OF PREG-NANCY	IRON			NITROGEN			PHOSPHORUS		
			Food	Feces	Balance	Intake	Outgo		Intake	Outgo	
							Urine	Feces		Urine	Feces
A 1	None, control	22	mg. 19.64	mg. 14.97	mg. 4.67	gm. 12.94	gm. 9.25	gm. 1.40	gm. 1.81	gm. 0.755	gm. 0.998
A 2	Wheat germ ash	23	27.04	19.94	7.10	13.19	9.24	1.42	2.53	0.930	1.066
A 3	Wheat germ	23	27.28	20.48	6.80	13.24	9.02	1.54	2.68	0.870	1.057
A 4	Wheat germ	24	26.03	19.22	6.81	12.94	8.82	1.68	2.44	0.810	1.085
A 5	None, control	24	20.13	16.68	3.45	12.19	9.10	1.39	1.70	0.784	0.884
A 6	Cod liver oil	25	21.43	16.92	4.51	12.21	8.62	1.42	2.17	0.749	0.900
A 7	Cod liver oil	25	22.42	20.46	1.96	12.25	8.66	1.64	1.95	0.812	0.999
A 8	Wheat germ and cod liver oil	26	28.03	20.14	7.89	12.97	8.64	1.66	2.67	0.888	0.984
A 9	None, control	27	21.02	15.38	5.64	13.25	9.40	1.39	2.46	0.878	0.826
B 1	None, control	31	18.88	16.61	2.27	10.19	8.01	1.27	0.91	0.736	0.920
B 2	Wheat germ ash	31	28.31	21.11	7.20	10.60	7.71	1.24	1.65	0.819	0.899
B 3	Wheat germ	32	32.14	23.34	8.80	10.76	7.59	1.31	1.86	0.792	0.870
B 4	Wheat germ	32	27.35	16.26	11.09	10.45	7.18	1.29	1.98	0.927	0.772
B 5	None, control	33	22.27	16.33	5.94	10.25	7.30	1.31	1.65	0.730	0.736
B 6	Cod liver oil	34	18.70	12.33	6.37	10.62	7.00	1.23	2.39	0.664	0.750
B 7	Cod liver oil	34	18.72	12.98	5.24	10.29	6.55	1.25	2.50	0.621	0.696
B 8	Wheat germ and cod liver oil	35	23.94	12.63	11.31	10.76	6.80	1.41	2.55	0.732	0.822
B 9	None, control	35	17.94	13.86	4.08	10.61	7.17	1.21	2.24	0.698	0.645
											0.325

<sup>1</sup> The week which included most of the 4-day period.

TABLE 2

*The effect of cod liver oil and wheat germ on the retention of calcium and magnesium during pregnancy (daily averages)*

SERIES AND PERIOD	SUPPLEMENT TO BASIO DIET	WEEK <sup>1</sup> OF PREG- NANCY	CALCIUM				MAGNESIUM			
			Intake	Outgo		Balance	Intake	Outgo		Balance
				Urine	Feces			Urine	Feces	
A 1	None, control	22	gm. 1.265	gm. 0.140	gm. 1.101	gm. 0.024	gm. 0.424	gm. 0.119	gm. 0.312	gm. —0.007
A 2	Wheat germ ash	23	1.326	0.141	1.106	0.079	0.554	0.126	0.417	0.011
A 3	Wheat germ	23	1.318	0.136	1.081	0.101	0.561	0.121	0.420	0.020
A 4	Wheat germ	24	1.314	0.132	1.081	0.101	0.551	0.118	0.428	0.005
A 5	None, control	24	1.310	0.123	1.000	0.187	0.441	0.115	0.298	0.028
A 6	Cod liver oil	25	1.277	0.145	0.991	0.141	0.443	0.114	0.311	0.018
A 7	Cod liver oil	25	1.285	0.163	1.057	0.065	0.453	0.097	0.366	—0.010
A 8	Wheat germ and cod liver oil	26	1.330	0.177	0.971	0.182	0.551	0.105	0.435	0.011
A 9	None, control	27	1.281	0.176	0.874	0.231	0.448	0.115	0.320	0.013
B 1	None, control	31	1.191	0.127	1.101	—0.037	0.377	0.117	0.275	—0.015
B 2	Wheat germ ash	31	1.215	0.132	1.008	0.175	0.482	0.124	0.337	0.021
B 3	Wheat germ	32	1.184	0.112	0.922	0.150	0.475	0.110	0.347	0.018
B 4	Wheat germ	32	1.198	0.160	0.857	0.181	0.468	0.109	0.296	0.063
B 5	None, control	33	1.174	0.102	0.871	0.201	0.369	0.118	0.245	0.006
B 6	Cod liver oil	34	1.153	0.119	0.894	0.140	0.377	0.098	0.253	0.026
B 7	Cod liver oil	34	1.186	0.120	0.822	0.244	0.379	0.088	0.256	0.035
B 8	Wheat germ and cod liver oil	35	1.205	0.098	0.894	0.213	0.474	0.079	0.361	0.034
B 9	None, control	35	1.149	0.095	0.775	0.279	0.371	0.072	0.248	0.051

<sup>1</sup> The week which included most of the 4-day period.

The storage ranged from nearly 2 mg. daily in one of the periods with added cod liver oil to more than 11 mg. daily in two different periods with added wheat germ. The highest retention represented 45 per cent of the intake while the average for the eighteen periods was 26 per cent of the amount ingested. The response to the addition of wheat germ was immediate in all instances.

Periods A 2 and B 2 show clearly, however, that most of the improvement in storage was due to the inorganic portion of the wheat germ represented by the ash. Rose and co-workers ('32, '34) have reported similar results from the ash and the organic portions of whole wheat and certain other cereals when measured by hemoglobin regeneration in the nutritional anemia of the rat, except that they associated the benefit with the bran portion of the whole grain. Doubtless some of the supplemental value of the ash was due to a greater supply of available iron, perhaps some to the supply of needed copper or other elements (Schultze et al., '34; Sherman, Elvehjem and Hart, '34).

The progressively greater storage in periods B 3 and B 4 indicated some benefit from the organic portion of the wheat germ, but corresponding periods in series A do not support this view. In periods A 8 and B 8, doubly supplemented, the improvement in storage represented approximately 100 per cent of the increase in iron intake, hence optimum utilization of the added iron. Another fact indicating that the supplementary effect of the wheat germ was due to more than its iron content was the high storage of period B 8 with wheat germ as contrasted to that of B 5, a control with the same iron intake, and the accomplishment of this retention through the reduction of fecal iron.

In either series the wheat germ in conjunction with the cod liver oil gave the best retention found. As will be shown later the same was true for nitrogen. Cod liver oil alone seemed to be without effect on the iron retention under the conditions of this experiment (periods A 7 and B 7).

Storage tended to be lower in series A than in series B in spite of reductions in intake in the latter. The daily averages were 5.42 and 6.93 mg. of iron, respectively. The advance of pregnancy may have been a factor in stimulating to greater iron utilization in the last series. On the other hand, the omission of half of the eggs in this series may have improved the retention by providing fewer sulfide radicals to divert both copper and iron from absorption according to the mechanism described by Sherman and co-workers ('34).

On the whole the storage of iron by this individual was much above that previously found for other gravid subjects. The average for this woman equalled the maximum previously recorded. For this woman the average intake during all periods was 23.37 mg. daily, as compared to 14.72 (Coons, '32) and 15.74 mg. (Coons et al., '35) for other groups, while the average retention of 6.18 by this case corresponded to 3.16 and 2.45 mg. daily for the other studies.

Some of the high quality of the diet might be attributed to the large quantity of peaches, 400 gm. daily, along with generous amounts of whole wheat, since the former have been found to be so potent for blood regeneration (Robschey-Robbins, '33). While these items may have contributed to the good storage found on the basic diet, the experiment showed that such storage could be further augmented by the wheat germ preparation.

The average daily fecal iron tended to remain constant (Coons, '32) from period to period except as influenced by marked changes in intake and these did not always alter the fecal output of iron. The iron content of dried feces varied widely, from 0.4196 to 0.8076 mg. per gram of dried stool, but was more consistent than found in this laboratory for different individuals on home diets for whom the range was five-fold, 0.2748 to 1.2840 mg. of iron per gram.

Some light on the question of iron utilization is gained from the similar curves for iron and nitrogen shown in figure 1. Elsewhere (Coons et al., '35) poor iron storage has been associated with deficient nitrogen economy. This is to

be expected wherever formation of whole blood is dependent upon exogenous supplies of both iron and nitrogen.

If the rate of iron retention accomplished by this subject throughout most of the last half of pregnancy is a desirable standard, then many of the balances reported heretofore were deficient and the estimated fetal needs of 4.75 mg. daily in the last trimester of gestation (White House Conference, '32) represents by no means the total needs of gravidity. The need for more data from controlled metabolic studies is obvious in view of the prevalence of anemias of dietary and gastro-intestinal origin particularly in pregnancy and infancy. One must keep in mind the fact that iron retention is only one step in the direction of iron utilization and of subsequent whole blood formation.

*Nitrogen.* Nitrogen retention was quite uniform throughout the experiment. The storage amounted to approximately 2 gm. of nitrogen daily in seventeen of the eighteen metabolic periods with an average of 2.15 gm. for all of both series. This was in accord with an observation previously noted that published data on nitrogen balances during pregnancy showed a definite tendency to a common storage around 2 gm. daily (Coons and Marshall, '34; Macy and Hunscher, '34). The influence of the advance of pregnancy was not enough to overcome the 20 per cent reduction in intake and maintain a level of storage in series B equivalent to that found in series A. The daily averages for the two were 1.97 and 2.27 gm., respectively.

Lowest retention occurred in the control periods, each supplement being associated with better storage, the wheat germ slightly more. Best retentions accompanied the use of both supplements simultaneously, but the differences were not marked. The better nitrogen storage observed during the periods with only the ash of the wheat germ added to the basic diet was related, doubtless, to the improved inorganic salt metabolism as manifested by better storage for all minerals studied (fig. 1, periods A 2 and B 2).

There was no evidence from these data that the benefits from the supplements were by way of increased absorption of nitrogen. Fecal nitrogen showed a utilization of 87 to 90 per cent of the nitrogen intake from period to period. This seemed to be a normal rate for such individuals (Coons et al., '35).

Probably the experimental diet, as planned, was less adequate in protein than in any other food essential. The nitrogen intake (table 1) was 12 to 13 gm. during series A and approximately 10.5 gm. during series B. It varied less than that of any other element studied in this supposedly constant diet, due, doubtless, to the fact that most of the nitrogen came from only two sources, milk and eggs, which tend to be more uniform in structure and nitrogen content than do other protein-containing foods such as meats.

Figure 1 shows that in period A 7, with the cod liver oil addition to the basic diet, the lowest storage in the series was found for every element studied. The nitrogen coefficient of digestibility was 86.6 per cent, which was as low as any found for the entire experiment. Evidence from original data indicated that although the number of stools was regular for this period, the percentage of moisture was higher and the average total dry feces per day was the largest found in any period of either series. The cause for this increased fecal output was not clear. However, as will be seen later, a higher urinary excretion also contributed to the lower balances recorded for calcium, phosphorus and magnesium, suggesting thereby some error in metabolism in general, not alone in absorption.

*Phosphorus.* Fluctuations in retention of phosphorus paralleled those for nitrogen when they differed from calcium (fig. 1). Supplements tended to improve the storage only slightly.

Retentions were better in series A with the higher intake than in series B. The daily averages were 0.338 and 0.267 gm., respectively, from corresponding average intakes of 2.147 and 1.783 gm. of phosphorus. The storage rates in periods B 1

and B 5, control periods on the lower diet, were inadequate for the total needs of gestation at that time. However, at the lower level of intake, 1.6 gm. daily, cod liver oil was more effective in improving the retentions than at intake levels of 2.0 gm. or over. As in previous studies the storage rates for phosphorus indicated that the total gestational needs for this element are more uniform throughout pregnancy than the requirement of calcium, magnesium or iron (Coons et al., '35).

In each series the control periods, A 9 and B 9, which followed the three consecutive cod liver oil periods, supported a rate of phosphorus storage equivalent in quantity to that in the preceding supplemented periods and that in spite of reductions in phosphorus intake. These findings suggest, among other things, a delayed effect of the cod liver oil which was not maximum even after 12 days of administration of 15 gm. daily. Such a retarded response may mean that the dosage was insufficient for prompt action or that the benefit to phosphorus utilization was secondary to the effect on calcium metabolism. The data for calcium retention, however, do not support the latter view.

The utilization of dietary phosphorus under the conditions of this experiment was better than was found for a group of Chicago women who had an average storage of 0.161 gm. on an intake of 1.431 gm. of phosphorus daily. However, the cod liver oil therapy was required to promote a phosphorus utilization equal to that shown by Oklahoma women under the influence of sunlight and with the same dietary level (periods B 6 to B 9). The latter had an average storage of 0.299 gm. daily on intakes of 1.632 gm. of phosphorus (Coons et al., '35) as compared to an average intake of 1.965 gm. and a storage of 0.302 gm. daily for the entire eighteen periods of this study. Data from these two investigations suggest that intakes of phosphorus as low as 1.6 gm. daily approach the minimum level of adequacy, even with reinforcement from cod liver oil or sunshine. Intakes around 2.0 gm. daily gave uniformly better retentions under the variety of conditions which prevailed in the two studies.

*Calcium.* Calcium balances, like those for iron, constituted one of the high points of interest in the original plans for this experiment. Final analysis revealed a storage much below the expected rate and with one negative balance.

The calcium intake (table 2) was only a little over 1 gm. of calcium daily and was 0.10 gm. less in the second series than in the first. At the intake level used and as planned poor storage had been demonstrated by women without access to potent sunshine (Coons and Blunt, '30) and with or without varying doses of cod liver oil added to self-chosen diets. On the other hand, at this same intake level good storage had been recorded for women of this section receiving a reasonable amount of sunlight (Coons et al., '35).

In the last group intakes of 1.2 gm. or less daily resulted repeatedly in retentions above 0.30 gm. of calcium daily. In the eighteen balance periods of the present study the retention did not at any time reach this figure, which represented the maximum daily deposit deemed necessary for the fetus alone. The diet for this subject possessed almost every advantage, except for calcium content, over the self-chosen diets of both groups previously studied.

The response to cod liver oil administration was slight when the storage of calcium was compared from period to period or with preceding control periods. On the other hand, in each series the highest calcium retention, like that of phosphorus, occurred in the control period following the cod liver oil therapy. Whether this represented a restorative after-effect of the therapy or was evidence of a delayed action of the supplement or of the cumulative effect of a subadequate dosage remains to be investigated. The urinary calcium remained constant for the last two periods of each series and reductions in fecal calcium provided the augmented retentions which marked the final control periods.

During the consecutive final months of gestation touched by the balances in this experiment the estimated daily fetal need for calcium ranged as follows: 0.055, 0.137, 0.225 and 0.306 gm. (Coons et al., '35). The corresponding average



storage rates by months for this subject were: 0.098, 0.155, 0.136 and 0.215 gm. daily. The 2 months with highest averages each included the 12 days of cod liver oil administration. For all eighteen periods the average storage was 0.152 gm. of calcium daily. Obviously the quantities retained were scarcely sufficient for the needs of the fetus alone.

Additional evidence of inadequate retention of calcium was that the level of storage in the second series was higher than in the first in spite of slight reductions in diet, and further, that there was a gradual increase in the rate of retention during each series, particularly the last. The highest storage found was in the final control period and on the lowest intake. Such increasing calcium storage coincident with the advance of late gestation has been interpreted in previous studies as indicating subadequate retention and failure to replenish maternal reserves prior to the heavy fetal demands of this period.

The tentative conclusion seems warranted, therefore, that under the conditions of this experiment 15 gm. of cod liver oil daily was not equivalent in its effect on calcium retention during human pregnancy to that of incidental but reasonable amounts of irradiation with the sunlight of this section during the spring and fall months. The need for similar observations on other individuals is obvious.

No effect of wheat germ on calcium metabolism was demonstrated by this experiment. During periods characterized by the wheat germ supplement alone retention fluctuated and was similar to that during the period containing the ash of wheat germ. On the other hand, it was not clear whether the better storage of periods A 8 and B 8 was due to cod liver oil alone or to a summed effect of both supplements, as for example through the improved phosphorus content of the diet.

An optimum acid-base balance of the diet for pregnancy has been described with reference to calcium retention (Coons et al., '34). Although the acid-base balance of this diet was not determined, the urine pH during this experiment ranged

close to that previously found associated with good calcium utilization (Coons et al., '35). Thus in series A the pH was 7.2 in all periods except A 2 when it was 7.4, and A 3 and A 4, when it fell to 7.0. Correspondingly in series B it was 7.1 to 7.2, except for 7.3 during the feeding of the ash of wheat germ, and 6.9 during the two periods with 30 gm. daily of unashed wheat germ.

Figure 1 shows that the curve for calcium storage was erratic and widely fluctuating, comparing in this behavior to that of iron and of magnesium. Retention varied ten-fold between the highest and lowest figures and as much as 100 per cent in consecutive periods with the same diet treatment. The curve for calcium retention was not related to that of any other mineral although it most resembled that for magnesium.

Urinary calcium, although fairly constant from period to period, was less in the second than in the first series, and constituted 10 to 12 per cent of the total output. Marked changes in storage were accomplished mainly through reductions in fecal calcium.

The calcium data of this experiment indicate the need for controlled studies of the effect of larger doses of cod liver oil, of a comparison between the effect of cod liver oil and potent sunshine on the same individual, and of the action of other sources of vitamin D, irradiated ergosterol and ultraviolet irradiation of the individual, on calcium and phosphorus metabolism to determine whether the benefits from these factors are similar to those derived from moderate uses of potent sunlight. The increasing data on the multiple nature of vitamin D are significant in this connection.

*Magnesium.* In spite of an apparently adequate intake of magnesium (table 2) three negative balances occurred, two others amounted to equilibrium and all were poor. An average daily intake of 0.455 gm., range 0.369 to 0.561, gave a retention of 0.018 gm. daily. This was a lower utilization than was found for the women receiving Oklahoma sunlight and storing an average of 0.063 gm. from an intake of 0.394

gm. daily. Cod liver oil improved the retention only slightly in series B. As with calcium, a higher storage was found immediately following the cod liver oil administration (period B 9). Increases in magnesium intake, as from wheat germ, also improved the storage only slightly and irregularly. Some influence of the progress of gestation on the demands for magnesium was indicated by the average storage for series A of 0.009 gm. daily and for series B of 0.027 gm., corresponding to intakes of 0.492 and 0.419 gm. daily.

Figure 1 also shows the relation of the curve of magnesium retention to that of calcium. In periods A 3, 5 and 9, also in periods B 3, 8 and 9, the calcium and magnesium curves vary in the same direction and in opposition to those of one or more other elements. They were diverse to each other only in periods B 5 and B 6, although the proportions in B 4 resemble those for phosphorus and iron more than for calcium.

Here again is evidence that magnesium metabolism is not antagonistic to that of calcium when the intake amounts to no more than is found ordinarily in dietaries (Coons et al., '35). The existence of common factors governing the utilization of calcium and magnesium had been assumed from previously reported instances of poor magnesium retentions associated with poor calcium storage, or in other cases parallel good retention of the two elements.

Urinary magnesium was fairly constant when compared to variations in intake but fluctuated with increases or decreases in retention. On the other hand, fecal magnesium rose with the intake augmented from wheat germ and fell with reversion to the basic diet. For these reasons urinary magnesium varied from 10 to 35 per cent of the total output of this element.

*General discussion.* The foregoing data show that even during a condition of fairly constant normal physiological demands, such as growth, and under the regimen of a presumably constant diet, the amounts of different elements retained varied from week to week. Calcium, iron and mag-

nesium retentions fluctuated more than did phosphorus and the last more than nitrogen. Similar variations in mineral metabolism had been demonstrated already by this laboratory for non-gravid women on a regulated diet (Reder and Coons). Under the conditions of this experiment such irregular metabolic behavior is not easily explained by variations in demand or supply of these elements.

Reference has been made to the varied composition of this supposedly constant diet. Some of the marked fluctuations would have seemed incredible after the care known to have been used in preparing and weighing the diets, had not earlier experiences predicted such. Attention is called to instances in which changes in output corroborated the analyses of the diets (compare iron, period B 8; phosphorus, period B 1). Marked increases aside from those due to the inorganic portion of the wheat germ were most frequent in the case of iron. Some of this was due to the ease of contamination of food with this element during preparation for the table. Part of it has been explained by the acid canned foods used. Table 1 shows that the peak of irregularity centered around period B 3, tapering away by the end of period B 5, hence probably represented simultaneous contamination from more than one food source. The phosphorus content of the diet varied next most, due probably to differences in the baker's mixes of whole wheat bread. Dietary calcium was less constant than was magnesium, while nitrogen was most constant of all. Usually the variations, even of nitrogen, were sufficient to have masked true differences in retention if composites for each day had not been analyzed completely. In common with the findings of MacKay and Butler ('35) and of Toscani and Reznikoff ('34) these data show that the analysis of samples of every food item from each experimental diet and period is an indispensable routine in the determination of biochemical balances.

## SUMMARY

The practicability of maintaining a human subject on a prescribed diet, constant in quality and quantity, for experimental purposes covering eighteen metabolic periods and 101 consecutive days of pregnancy has been demonstrated.

Cod liver oil and a wheat germ preparation were used to supplement a basic diet in order to study the effect on the retentions of calcium, phosphorus, magnesium, iron and nitrogen under strict control of activities and environmental conditions.

The basic diet supported good retention of iron, but excellent storage was accomplished with the aid of the wheat germ preparation. The results were traced largely to the ash of the preparation rather than to the organic portion. The latter exerted a slight influence. Nitrogen retention was also improved by this supplement. Cod liver oil further supplemented the wheat germ in its effect on nitrogen and iron retention.

Cod liver oil to the extent of 15 gm. daily improved the retention of calcium, phosphorus and magnesium only slightly and irregularly. It did not raise the calcium and magnesium storage to the level previously recorded for a group of gravid women who had access to reasonable amounts of the sunshine of Oklahoma. The rate of calcium retention did not cover the fetal demands for the time involved.

Evidence of the influence of the progress of gestation on the rates of retention of the elements studied was strongest for calcium, iron and magnesium. The curves of retention for nitrogen most resembled that for iron, while calcium and magnesium of the minerals were most consistently related.

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A CORRECTION TO THE PAPER ENTITLED:  
THE FOOD VALUE OF ETHYL ALCOHOL

H. H. MITCHELL

In the paper entitled: "The food value of ethyl alcohol" that appeared in *The Journal of Nutrition* for September, 1935 (vol. 10, pp. 311-335), reference was made (p. 330) to a determination of the specific dynamic effect of starch when fed to rats reported by Kriss, Forbes and Miller in a paper that appeared in a previous number of the journal (vol. 8, pp. 509-534). Believing that the specific dynamic effect of starch (and of the other nutrients fed in these experiments) had been computed from heat productions which had been reduced in toto to a constant rat weight of 100 gm., the values of Kriss, Forbes and Miller were recomputed on the assumption that only the basal heat varies directly with the body surface.

Since the publication of the alcohol article, Doctor Forbes has called the author's attention to the fact that the specific dynamic effects were in fact computed from heat production values corrected for differences in body size only with reference to the basal fraction of heat. Hence, the recomputed values of the author, appearing in the text and in footnote 3 on page 331, are in error.

The author regrets exceedingly that he was misled by the brief description of the method of weight correction used by Kriss, Forbes and Miller, as given in their article (p. 514) and in the immediately following one by Forbes, Kriss and Miller (pp. 543 and 545), and by the manner in which the corrected heat production values were expressed, i.e., as hourly heat productions "per 100 gm. of empty body weight."

It need only be said in addition that neither the results of the alcohol experiments nor the conclusions derived from them are in any way impaired by this error.





# THE FOOD VALUE OF ETHYL ALCOHOL

H. H. MITCHELL

*Division of Animal Nutrition, University of Illinois, Urbana*

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Although the ability of the animal body to absorb and to oxidize ingested ethyl alcohol was early established, the physiological value of the energy thus liberated is still uncertain, in spite of numerous experimental contributions to the subject. Space will not permit a comprehensive review of this voluminous literature; it must suffice to refer to some of the most recent work, and only as it bears on the problem studied in the investigation to be reported here.

The utmost confusion prevails in regard to the question whether the energy liberated by the oxidation of alcohol is necessarily dissipated as heat (specific dynamic effect), or whether all or part of it is available for physiological work, thus sparing such nutrients as carbohydrates and fats, the food value of which is well known. Confirming many earlier experiments, notably that of Higgins ('17), Bornstein and Loewy ('27) observed no appreciable increase in the basal metabolism of human subjects after the ingestion of 25 to 30 gm. of alcohol by mouth, although rapid absorption of the alcohol was demonstrated by blood analysis, and appreciable oxidation by the observed depression of the respiratory quotient. Somewhat later, Lundsgaard ('31) secured increases as high as 26 per cent in the basal metabolism of dogs by the ingestion of 15 gm. of alcohol. Using rabbits as subjects, Kanai ('33) noted variable effects of alcohol on the basal metabolism, ranging from an initial marked depression followed in 1 or 2 hours by a slight increase, to a prolonged

but slight and irregular increase over a period of 6 hours. Terroine and Bonnet ('29), from a series of experiments on men, rabbits, and pigeons, conclude that alcohol is uniquely and solely thermogenic. If the experiment was performed at high temperatures, from 92 to 97 per cent of the energy of the administered alcohol not appearing in the urine was accounted for by the excess heat produced above the basal metabolic rate. Only if the experiment was performed at temperatures below the critical, was the basal heat production unaffected by alcohol ingestion, a result interpreted as a sparing of body nutrients by the heat of combustion of the alcohol.

The investigations of Mellanby ('19), Onlow ('24), Nicloux ('32), and Widmark ('34), mainly concerned with the rate of disappearance of ingested alcohol from the blood, support the conclusion that this rate is largely independent of the amount of alcohol consumed or of the kinds and proportions of nutrients undergoing simultaneous oxidation. In fact, the recent contributions of Carpenter, Lee and Burdett ('33) and of Nyman and Palmlov ('34), both upon human subjects, indicate clearly that the rate of disappearance of alcohol from the blood is unaffected by muscular activity (see also Carpenter, '33). Of the same significance are the experimental results reported by Canzanelli, Guild and Rapport ('34), to the effect that the R.Q. of the excess metabolism of work in dogs is unaffected by the ingestion of alcohol, even at times when the alcohol is exerting its characteristic depressing effect upon the R.Q. These investigators conclude definitely that "ethyl alcohol cannot be used as a source of oxidative energy for muscular exercise." However, this conclusion may be a hazardous interpretation of purely negative evidence.

The preponderance of recent experimental work thus appears to favor the view that the energy of ingested alcohol, although completely liberated within the animal body except for insignificant losses through the kidneys (Kionka and Haufe, '28) and lungs (Carpenter, Lee and Burdett, '33) has an extremely limited field of usefulness in metabolism. Shapiro's ('35) experiments afford further evidence of the

same significance, since she showed with rats that ethyl alcohol is distinguished from glucose by its inability to spare body protein during fasting, or to induce a storage of glycogen in the liver, or to exert a ketolytic action following the simultaneous ingestion of diacetic acid, except when a stimulation of protein catabolism occurred; in this case the ketolytic effect was probably exerted by the extra quota of amino acids drawn into the metabolizing mixture.

An attempt to determine the food value of alcohol by feeding experiments upon rats was made by Richter ('26), who found that the rat could tolerate for 3 to 7 months as much as 8 to 16 per cent solutions of alcohol as its steady fluid supply without intoxication, but with a definite decrease in spontaneous activity as measured in revolving cages. The alcohol consumed was estimated to represent 22 to 29 per cent of the total energy intake; the rats consuming these amounts of alcohol ate 17 to 63 per cent less food than the controls which drank water, grew just as rapidly, and reached the same body weight at maturity as the controls. It was concluded that "in the rat alcohol not alone replaces isodynamic quantities of food in maintaining energy balance, as was demonstrated by Atwater and Benedict, but also that it is used for growth and development." Of much the same significance are the observations of Ariyama ('33) that alcohol added to a high fat diet improved the growth rate of rats, the optimum concentration being 5 per cent. On analysis of the carcasses of the rats, it was found that the addition of alcohol to the fat diet favored the deposition of fat in the body, in particular in the liver.

It thus seems difficult to obtain a clear picture of the food value of ethyl alcohol from the evidence available in the literature. Some of the evidence must be discounted because of obvious uncertainties in significance. For example, the failure of Bornstein and Loewy to detect a specific dynamic effect of alcohol in human subjects may have been the result of a failure to place his subjects in the post-absorptive state before administering the drug. The reputedly basal metabolism measurements were made 11 to 13 hours after the last

meal, when the R.Q.'s were as high as 0.91 to 0.95. Also Richter's conclusion that alcohol replaces isodynamic quantities of food nutrients in metabolism, takes no account of the observed depression in muscular activity induced by alcohol, nor of the possibility that the gains in weight by test and control rats may have had quite different caloric values. The fact that the experiments were carried out in a room maintained at temperatures ranging from 20° to 22°C., which are considerably lower than the critical temperature of the fasting rat (28° to 30°C.), justifies some suspicion that the alcohol was sparing carbohydrates and fats merely (or in part) because it was providing heat for body temperature maintenance, not because it was furnishing energy for the various forms of physiological work. In Ariyama's experiments, the failure to observe the food intakes of his rats complicates the interpretation of his results with reference to the extent to which alcohol serves as a food. But these, or other, reasonable criticisms are not capable of reconciling all of the apparent conflicts of evidence. The picture is still blurred in many of its aspects.

Evidently there is still a need for further evaluations of ethyl alcohol as a food. The problem is of interest from a number of standpoints, not the least of which is the apparently normal presence of alcohol in body tissues and fluids (Gettler, Niederl and Benedetti-Pichler, '32), and its possible significance as an intermediary metabolite in carbohydrate metabolism (Shapiro, '35). The practical importance of the problem relates to the nutritive evaluation of alcoholic beverages. Attempts at such evaluation must (in the absence of any tenable alternative) ascribe to alcohol, rightly or wrongly, its full energy value of 7.11 calories per gram, and this is done in the medical literature. Thus, Christie ('32) states that "spirituous liquors owe their fat-forming qualities to the alcohol which they contain, and also in some instances to carbohydrate," and he discusses the importance of such beverages in the treatment of obesity and malnutrition.

The investigation reported below is an attempt to furnish further information on the food value of ethyl alcohol, following in general the procedure of Richter, but with certain refinements in technic suggested by a study of his experimental results. The general plan of the experiments has been to feed pairs of rats equal quantities of a good basal diet and to give to one rat of each pair variable supplements of 95 per cent alcohol. The nutritive effects of the alcohol were measured by determining the growth rates of pair mates over a number of weeks of this regime and by investigating the nitrogen and energy contents of the tissues formed from an analysis of the carcasses. The observed effects of alcohol were compared with the nutritive effects of sucrose determined in an entirely similar fashion, supplements of sucrose replacing supplements of alcohol. The comparative effects of alcohol and sucrose supplements upon the digestive processes were also studied by chemical examination of the feces of such paired rats.

To minimize the narcotic effects of the alcohol supplements, they were added to the basal diet rather than to the drinking water as in Richter's experiments. It has been abundantly proven that the rate of absorption of alcohol is definitely depressed by the simultaneous ingestion of food (see for instance, Mellanby, '19). In one series of experiments the rats were placed in revolving cages equipped with revolution counters, so that the alcohol supplements could be increased in size only as long as voluntary activity was not depressed. In a later series of experiments, the rats were confined in every small compartments in the hope of greatly restricting the energy expenditure on muscular activity and of minimizing any differential expenditure between test and control rats. In all experiments the temperature of the room in which the rats were kept was maintained at 80°F. or above in so far as possible. Since the first experiments were performed during warm or hot weather, this object was easily attained, but in the later and, in other respects, more successful feeding experiments, extending into September, it was not possible

to do so consistently, since no temperature control during the night was feasible. However, in the 42 days of this test, the night temperature dropped below 75° only ten times, and below 70° only once. By maintaining temperatures above the critical, it was hoped to distinguish any true nutritive value of the supplement from its ability merely to supply heat to the animal body.

In the first experiments with revolving cages, the alcohol was added to a solid diet, containing 25.5 per cent of whole egg protein and in all other respects capable of promoting rapid growth in rats. While these experiments, involving sixteen pairs of rats, provided clear indications that the energy of alcohol is available for growth, they were unsatisfactory because a variable evaporation of the alcohol from the food dishes undoubtedly occurred. The rats receiving the alcohol supplements were observed soon to defer the consumption of their food until evening, presumably to avoid the alcohol flavor. Under these conditions it was not surprising that in some pairs the growth-promoting effect of the alcohol was not observed. The results are hence statistically inadequate to establish a definite conclusion.

In the final tests, a liquid food, whole fresh milk, was used; the milk was fed in glass water fountains, such as Richter used. One rat in each pair received in its milk an alcohol supplement at the rate of 0.03 cc. per cubic centimeter of milk for the first 9 days of feeding, and then until the termination of the experiment at the rate of 0.04 cc. per cubic centimeter of milk. The alcohol supplement contained 92 per cent by weight of alcohol, 1 cc. having a heat of combustion of 5.29 calories. To prevent the development of anemia, the milk was mineralized by the addition of iron, copper, and manganese salts, according to the directions of Kemmerer, Elvehjem, Hart and Fargo ('32). As thus administered, the surface of alcoholized milk exposed to the air was so small that evaporation of alcohol was minimized, if not prevented for all practical purposes. The volume of milk consumed by pair mates was equalized throughout the test, which lasted 6 weeks.

The activity of the rats was greatly restricted by confining them in small compartments formed by bisecting with a small-mesh screen circular cages measuring only 6 inches in diameter and 7 inches high, each cage thus accommodating a pair of rats. The bottoms of the cages were made of  $\frac{1}{2}$ -inch mesh wire screening in order to minimize coprophagy.

The rats in the sugar experiment were later fed in the same cages and received also mineralized milk, the consumption of milk by pair mates being also equalized. In this experiment, one rat in each pair received a supplement of sucrose which, except for the first few days, amounted to 2.5 gm. daily, added to the milk in a 50 per cent water solution. The sucrose used contained 3.937 calories per gram by actual combustion in the bomb calorimeter. Since evaporation of volatile substances was a matter of no importance here, the milk and added supplements were offered in open glass dishes held securely in place to prevent spilling. This experiment was continued for 36 days.

The total extra energy consumed by the test rats in these two experiments was nearly the same, averaging 307 calories in the alcohol experiment and 357 calories in the sucrose experiment.

At the termination of each of these experiments the rats were killed with ether, their body lengths from nose to root of tail measured, and the empty weights determined by opening the abdomen and removing the contents of the alimentary tract. The carcasses were then frozen solid and in the frozen condition ground finely in a hand mill. The samples thus prepared were then submitted to chemical analysis. In the alcohol experiments only the nitrogen and gross energy content of the rat carcasses were determined, the former by the Kjeldahl method and the latter by combustion in a Parr oxygen bomb calorimeter. The rats in the sucrose experiments were analyzed for dry matter and ether extract in addition.

The significant results of these two experiments are summarized in tables 1 and 2.



TABLE 1  
*Results of the alcohol experiment: 42 days*

RAT NO. AND SEX	TOTAL MILK CONSUMED	TOTAL ALCOHOL CONSUMED	INITIAL BODY WEIGHT	FINAL BODY WEIGHT	GAIN IN BODY WEIGHT	FINAL BODY LENGTH	EMPTY BODY WEIGHT	COMPOSITION OF EMPTY CARCASS		TOTAL NITROGEN IN CARCASS	TOTAL ENERGY IN CARCASS
								Nitrogen	Gross energy		
	cc.	cc.	gm.	gm.	gm.	mm.	gm.	per cent	cal. per gm.	gm.	cal.
77 m	1475	0	43	114	71	181	107	3.14	2.10	3.37	226
78 m	1475	56.8	42	141	99	185	123	3.11	2.59	3.84	320
79 m	1430	0	41	114	73	181	106	3.05	2.19	3.24	232
80 m	1430	55.1	42	144	102	192	135	2.98	2.29	4.04	310
81 f	1335	0	45	99	54	175	92	3.18	2.36	2.92	217
82 f	1335	51.2	39	125	86	182	116	3.00	2.28	3.49	265
83 f	1375	0	36	104	68	173	96	3.24	2.15	3.10	206
84 f	1375	53.2	36	123	87	179	117	2.97	2.29	3.47	268
85 m	1655	0	40	138	98	191	129	3.01	2.29	3.89	295
86 m	1655	63.6	40	160	120	195	152	3.06	2.33	4.64	353
87 m	1825	0	38	158	120	193	147	2.61	2.29	3.83	336
88 m	1825	70.3	38	167	129	198	159	2.93	2.36	4.66	375
89 m	1675	0	37	137	100	189	125	3.00	2.23	3.75	279
90 m	1675	64.1	39	163	124	198	153	3.07	2.36	4.69	361
91 f	1340	0	43	93	50	172	88	3.33	2.01	2.93	177
92 f	1340	50.5	40	116	76	179	108	2.95	2.31	3.18	249

TABLE 2  
Results of the sucrose experiment: 36 days

RAT NO. AND SEX	TOTAL MILK CONSUMED	TOTAL SUGAR CONSUMED	INITIAL BODY WEIGHT	FINAL BODY WEIGHT	GAIN IN BODY WEIGHT	FINAL BODY LENGTH	EMPTY BODY WEIGHT	COMPOSITION OF EMPTY CARCASS				TOTAL NITROGEN IN CARCASS	TOTAL ENERGY IN CARCASS
								Moisture	Nitrogen	Fat	Gross energy		
	cc.	gm.	gm.	gm.	gm.	mm.	gm.	per cent	per cent	per cent	cal. per gm.	gm.	cal.
331 m	1215	0	42	123	81	183	113	67.3	3.12	9.35	1.96	3.52	221
332 m	1215	90.7	42	153	111	192	142	64.9	3.02	11.52	2.13	4.30	304
333 f	1582	0	51	114	63	174	107	61.1	3.07	15.52	2.39	3.28	256
334 f	1582	90.7	49	138	89	184	131	59.3	2.96	18.78	2.87	3.89	376
335 m	1850	0	48	145	97	185	134	60.9	3.01	15.82	2.49	4.03	334
336 m	1850	90.7	49	167	118	193	157	62.6	3.00	14.60	2.39	4.71	375
337 m	2110	0	57	165	108	193	153	61.3	3.04	15.36	2.49	4.65	381
338 m	2110	90.7	55	219	164	210	202	61.8	3.04	16.27	2.56	6.12	515
339 f	1757	0	53	129	76	174	119	58.2	2.93	21.31	2.88	3.49	342
340 f	1757	90.7	53	164	111	189	153	62.1	3.00	15.92	2.45	4.61	376
341 f	1765	0	56	120	64	175	113	62.1	2.94	14.57	2.43	3.32	275
342 f	1765	90.7	58	160	102	193	151	60.0	2.95	18.80	2.76	4.45	417
343 m	2055	0	52	168	116	193	152	63.0	3.01	14.42	2.42	4.58	368
344 m	2055	90.7	51	196	145	202	181	59.6	2.80	18.72	2.71	5.07	492
345 f	1491	0	41	111	70	172	101	62.6	3.05	13.97	2.40	3.10	244
346 f	1491	90.7	42	141	99	183	133	58.9	2.96	19.79	2.79	3.94	372
347 m	1642	0	43	135	92	186	121	64.3	3.14	12.57	2.23	3.80	270
348 m	1642	90.7	42	164	122	193	152	63.5	3.07	14.39	2.36	4.66	359

Among the eight pairs of rats in the alcohol test, the rat receiving the alcohol supplement put on the larger live weight gain in all pairs, and attained a greater final empty weight and a greater final body length. From these figures, the growth-promoting value of ethyl alcohol is clearly demonstrated. The percentage of nitrogen in the empty carcasses of control and test rats did not differ in a consistent fashion and averaged very nearly the same, i.e., 3.07 and 3.06. However, the absolute content of nitrogen was greater in all pairs in the carcass of the alcohol rat, the average difference amounting to 0.623 gm. Thus, to this average extent the alcohol supplement promoted nitrogen storage.

The energy content of the carcasses of pair mates, expressed in calories per gram, was greater for the alcohol rat in all pairs but one. A statistical analysis of these paired differences according to the method of 'Student' ('08) indicates a probability of only 0.024 that they were produced by a fortuitous combination of factors common to both pair mates; the average of the differences was 0.149 calorie per gram and their standard deviation, 0.163. Evidently the alcohol produced gains in weight with a greater content of fat than those produced on the basal diet alone. The total excess energy in the carcasses of the alcohol rats, evident in each of the eight pairs, averaged 66.6 calories. This value represents the average recovery of the energy of the alcohol fed in the increase in body substance. For each gram of extra nitrogen stored by the alcohol rats there was an extra deposition of 107 calories of energy.

The sucrose experiment, summarized in table 2, was not designed to demonstrate the food value of sucrose, since this is fully known. Its purpose was twofold, first, to test the method of procedure used in the alcohol experiment with reference to its ability to account for the energy of supplements to the basal diet, and, second, to afford a comparison of the food value of alcohol with that of a common food nutrient of high known value. As would be expected, the rat in each of the nine pairs that received the sucrose supplement

gained in weight more, attained a greater empty weight and body length, and stored more nitrogen and energy in their bodies. The extra nitrogen and energy averaged 0.887 gm. and 99.4 calories, respectively, quantities 42 and 49 per cent greater than the corresponding extra storages observed in the alcohol experiment. The ratio of the two was practically the same as in the alcohol experiment, i.e., 112 calories per gram of nitrogen, as compared with 107.

The carcasses of the sucrose rats contained on the average somewhat less moisture, 61.41 as compared with 62.31 per cent for the controls, somewhat less nitrogen, 2.98 against 3.03 per cent, somewhat more fat, 16.53 against 14.77 per cent, and somewhat more energy per gram, 2.56 against 2.41 calories, but these differences are none of them statistically significant, although the latter two are highly suggestive of an effect of the extra sucrose calories consumed. The probability that chance produced these latter differences are 0.081 and 0.077, respectively.

Before a complete accounting of the extra energy of alcohol and of sucrose provided in the above experiments is possible, it is necessary to consider the effects of these addenda upon the fecal excretions. For this purpose, eight pairs of rats were used. In an initial period the pairs were fed whole milk, the intake of pair mates being equalized. One rat in each pair was given daily 2.5 gm. of sucrose in addition. After a period varying from 11 to 14 days, during which the feces were collected, the sucrose dosage was discontinued, and the control rat was then given a supplement of 2 cc. of ethyl alcohol daily. The collection periods on this regime started from 2 to 9 days later, and continued for 9 to 13 days. The feces were marked by administering to each rat on the first day of each collection period, and also the first day following the termination of the period, a mixture composed of 100 mg. of either  $\text{Fe}_2\text{O}_3$  or  $\text{Cr}_2\text{O}_3$  and 1 gm. of glucose. The feces thus collected were dried, sieved to remove the hair, and analyzed for total nitrogen; the heats of combustion were determined in the bomb calorimeter. The results of this test are assembled in tables 3 and 4.

TABLE 8  
*The effect of sucrose on digestion*

RAT NO. AND SEX	AVERAGE BODY WEIGHT	MILK CONSUMED DAILY	SUCROSE CONSUMED DAILY	TOTAL GAINS IN BODY WEIGHT	FECAL CONSTITUENTS EXCRETED DAILY			NITROGEN IN DRIED FEACES	ENERGY IN DRIED FEACES
					Dry matter	Nitrogen	Gross energy		
	gm.	cc.	gm.	gm.	mg.	mg.	cal.	per cent	cal. per gm.
383 m	131	40	0	17	351	15.9	1.70	4.52	4.84
384 m	140	40	2.5	38	361	16.8	1.94	4.64	5.37
385 m	130	40	0	16	347	17.2	1.56	4.95	4.50
386 m	144	40	2.5	30	402	21.5	2.12	5.35	5.28
395 m	107	40	0	28	281	13.0	1.31	4.64	4.67
396 m	126	40	2.5	47	368	19.0	2.02	5.16	5.48
397 m	106	40	0	26	327	13.9	1.74	4.26	5.32
398 m	112	40	2.5	37	388	19.9	2.05	5.13	5.28
399 m	115	40	0	16	354	15.6	1.80	4.41	5.08
400 m	131	40	2.5	31	433	22.7	2.46	5.25	5.68
403 f	110	43	0	16	384	19.8	1.81	5.15	4.72
404 f	125	43	2.5	33	344	18.1	1.76	5.26	5.12
409 m	104	40	0	15	344	17.4	1.52	5.07	4.41
410 m	121	40	2.5	35	383	18.5	1.95	4.84	5.09
411 f	101	40	0	17	416	18.7	1.85	4.50	4.44
412 f	107	40	2.5	29	464	25.9	2.32	5.58	5.00

TABLE 4  
*The effect of alcohol on digestion*

RAT NO. AND SEX	AVERAGE BODY WEIGHT	MILK CONSUMED DAILY	ALCOHOL CONSUMED DAILY	TOTAL GAINS IN BODY WEIGHT	FECAL CONSTITUENTS EXCRETED DAILY			NITROGEN IN DRIED FEACES	ENERGY IN DRIED FEACES
					Dry matter	Nitrogen	Gross energy		
	gm.	cc.	cc.	gm.	mg.	mg.	cal.	per cent	cal. per gm.
383 m	171	49	2	29	483	24.1	2.24	4.99	4.63
384 m	171	49	0	19	520	24.2	2.53	4.65	4.86
385 m	175	50	2	39	501	27.3	2.28	5.45	4.55
386 m	176	50	0	17	521	25.8	2.56	4.95	4.92
395 m	154	47	2	31	381	18.2	1.82	4.78	4.78
396 m	163	47	0	16	396	18.5	1.94	4.68	4.89
397 m	166	50	2	31	535	26.9	2.92	5.02	5.46
398 m	153	50	0	15	466	22.0	2.32	4.72	4.97
399 m	159	48	2	35	466	22.3	2.36	4.79	5.07
400 m	160	48	0	17	550	26.4	2.77	4.80	5.04
403 f	148	50	2	28	478	27.2	2.13	5.68	4.46
404 f	150	50	0	12	511	26.8	2.39	5.25	4.67
409 m	155	50	2	44	346	17.9	1.52	5.18	4.40
410 m	156	50	0	22	470	22.1	2.23	4.71	4.75
411 f	146	45	2	31	384	23.8	1.73	6.20	4.51
412 f	131	45	0	9	451	24.1	1.96	5.35	4.34

The rats receiving the alcohol or the sucrose supplements gained in weight much faster than their pair mates. However, the effects of the two supplements on the fecal excretion were quite dissimilar. The feces produced on the sucrose supplement, as compared with the control feces, contained significantly larger amounts of dry matter per day (Mean = + 42.4 mg., standard deviation = 38.24, Probability = 0.011), of nitrogen ( $M = + 3.86$  mg.,  $s = 3.13$ ,  $P = 0.007$ ), and of energy ( $M = + 0.416$  calories,  $s = 0.235$ ,  $P = 0.0011$ ), and contained also a significantly greater percentage of nitrogen ( $M = + 0.464$  per cent,  $s = 0.421$ ,  $P = 0.011$ ) and a greater energy content per gram ( $M = + 0.540$  calorie,  $s = 0.252$ ,  $P = 0.0004$ ). Probably this excess fecal excretion does not represent a depression in the digestibility of the nutrients of the milk, but rather an increased production of metabolic products in the feces. This conclusion is based upon the fact that the ratio of the extra fecal nitrogen (3.86 mg.) to the extra dry matter consumed daily in the sucrose supplement (2.5 gm.), 1.54 mg. per gram, is of the same order of magnitude as the ratio of total fecal nitrogen to consumed dry matter on a diet containing no nitrogen or no indigestible nitrogen (Schneider, '35). The extra fecal energy produced per gram of sucrose supplement is 0.167 calories. The author ('34) has obtained elsewhere an average ratio of 0.164 calorie of fecal energy per gram of a carbohydrate diet consumed; the diet contained no appreciable amount of indigestible nutrients.

In contrast to the sugar, the alcohol supplement was associated with a lower average excretion of fecal dry matter, fecal nitrogen, and fecal energy. However, the average decrease in fecal nitrogen is obviously insignificant, while that of dry matter ( $M = - 38.9$  mg.,  $s = 53.2$ ,  $P = 0.048$ ) and of energy ( $M = - 0.212$  calorie,  $s = 0.348$ ,  $P = 0.076$ ) are on the borderline of significance, although in each case only one of the eight pairs presents a contrary result. These apparent effects of alcohol can be interpreted only as an improvement in the digestion of the basal diet of milk, probably brought about by a stimulation of the digestive glands (Newman and

Mehrtens, '32-'33). It is a matter of considerable interest that no evidence of an increased excretion of metabolic fecal products as a result of alcohol ingestion was secured. The percentage of nitrogen in the feces of the rats receiving the alcohol supplements was significantly greater than that of

TABLE 5  
*The accountable disposition in the animal body of the energy of alcohol*

PAIR NO.	EXTRA ENERGY IN ALCOHOL	EXTRA BASAL HEAT	EXTRA ENERGY BALANCE	EXTRA FECAL ENERGY	TOTAL ENERGY OF ALCOHOL ACCOUNTED FOR	
					Calories	Per cent
	<i>cal.</i>	<i>cal.</i>	<i>cal.</i>	<i>cal.</i>		
1	300	58	94	—6	146	49
2	291	56	78	—6	128	44
3	271	23	48	—5	66	24
4	281	37	62	—6	93	33
5	336	36	58	—7	87	26
6	372	53	39	—7	85	23
7	339	54	82	—7	129	38
8	267	20	72	—5	87	33

TABLE 6  
*The accountable disposition in the animal body of the energy of sucrose*

PAIR NO.	EXTRA ENERGY IN SUCROSE	EXTRA BASAL HEAT	EXTRA ENERGY BALANCE	EXTRA FECAL ENERGY	TOTAL ENERGY OF SUCROSE ACCOUNTED FOR	
					Calories	Per cent
	<i>cal.</i>	<i>cal.</i>	<i>cal.</i>	<i>cal.</i>		
1	357	53	83	15	151	42
2	357	45	120	15	180	50
3	357	40	41	15	96	27
4	357	94	134	15	243	68
5	357	81	34	15	130	36
6	357	74	142	15	231	65
7	357	67	124	15	206	58
8	357	50	128	15	193	54
9	357	58	89	15	162	45

the feces of the control rats ( $M = + 0.372$  per cent,  $s = 0.247$ ,  $P = 0.0026$ ), but obviously the energy per gram was closely the same.

In tables 5 and 6 an attempt is made to account for the extra energy of the alcohol and sugar supplements fed in the



two growth experiments summarized in tables 1 and 2. In the second column of each table will be found the total energy of the supplements. In the next column is an estimate of the extra basal calories of the rats receiving the supplement over that of their controls receiving the same amounts of milk. These values are the summation of values computed weekly from the average weekly weights, the rats having been weighed daily. From the average body weights per week, the average surface areas were calculated by Lee's ('29) formula  $S_{\text{cm}^2} = 12.54 W_{\text{gm.}}^{0.60}$ . The basal metabolism was assumed to equal 760 calories per day per square meter of body surface (Smuts, '35). The extra energy balances, listed in column 4 of each table, represent the extra energy stored in the carcasses of the rats receiving supplements over that of their pair mates, and are computed from the values given in the last columns of tables 1 and 2. The extra fecal energy excreted is a positive value in the case of the sucrose, estimated at 0.167 calorie per gram of sucrose supplement, and a negative value for the alcohol, estimated at  $-0.106$  calorie per cubic centimeter of alcohol supplement, expressing the fact that sucrose increases the excretion of metabolic fecal products, and the probability that alcohol increases the digestibility of the energy of the basal diet. Adding these various factors together and expressing the sum of the accounted for energy as a percentage of the energy value of the supplement gives the values in the last columns of tables 5 and 6. From 23 to 49 per cent of the energy of the alcohol was thus recovered, the average being 33.8 per cent. For the sucrose the corresponding percentages range from 27 to 68, and average 49.4.

The recovery of the energy of the sucrose supplements thus averaged considerably higher than the recovery of the energy of the alcohol supplements. But individual rats in both experiments exhibited great divergencies from their respective means, so that much over-lapping of individual percentage recoveries may be noted by comparing the values in tables 5 and 6. It becomes a statistical problem, therefore, to deter-

mine whether the sucrose supplements were actually better utilized than the alcohol supplements. Fisher ('28, p. 107) has proposed a modification of the 'Student' method for application to small groups of unpaired observations. Applying this method to the present instance, the mean difference between the alcohol and the sucrose groups is 15.6 per cent, the standard deviation is 11.74,  $t = 2.735$ ,  $n = 15$ , and  $P = < 0.02$ . This probability that a fortuitous combination of factors common to both groups may have accounted for the average group difference is so small that it may be neglected. We may conclude, therefore, that a more complete recovery of the energy of sucrose was secured than of the energy of alcohol.<sup>1</sup>

The recovery of less than 50 per cent of the energy of the sucrose supplements implies that the specific dynamic effect of sucrose is equivalent to as much as 50 per cent of its total energy content, a seemingly excessive figure. But the average recovery of 49.4 per cent of the sucrose energy is minimal, since the energy expenditure for muscular activity by the sucrose rats would be greater than that of their control mates, assuming the same degree of activity for both, because the sucrose rats were heavier throughout the experiment. This unmeasurable factor is probably of greater importance in the sucrose experiment than in the alcohol experiment, since the sucrose rats gained an average of 6.5 gm. per week more than their control mates, while the alcohol rats gained weekly only 3.9 gm. more than their controls. Also, the presumably greater activity expenditure of the alcohol rats than that of their pair mate controls may well have been entirely nullified if the alcohol supplements depressed the degree of activity on account of their potential narcotic effect. In this connection it must be remembered, first, that muscular activity of all rats was presumably subnormal because of the restricted con-

<sup>1</sup> However, an alternative explanation is that the actual alcohol intake of the rats receiving alcohol supplements was appreciably less than the alcohol offered, due to the evaporation of alcohol from the exposed surface of the milk in the glass fountains. This area equals about 1.75 cm.<sup>2</sup> It would be extremely difficult to investigate this possibility experimentally. It seems to the writer improbable that this error would be a serious one.

finement, and, second, that the alcohol rats, although observed particularly, never appeared to be apathetic.

It seemed possible that the method of measuring the nutritive energy of sucrose and alcohol by adding them in relatively small amounts to a basal diet possessed a constant error, in the sense that a certain minimum addition would be required before any observable effect at all was obtained. Hence, a series of auxiliary paired-feeding tests were performed with sucrose to determine how large a daily supplement was required to produce a statistically significant effect on body weight and body length with eight pairs during a feeding period of 6 weeks. Daily supplements of 100, 200, and 400 mg. of sucrose were given to one rat in each pair, using a well-balanced diet, and in a later test, the largest supplement was used with a basal diet of mineralized milk.

The rat receiving the 100-mg. supplement of sucrose made the greater gain in weight in only four of the eight pairs, and attained the greater body length in six pairs. The average difference in total gain between pair mates was only 0.75 gm. in favor of the sucrose rats, and the average difference in final body length was only 2.25 mm., again in favor of the sucrose rats. Both differences were obviously insignificant statistically.

The 200-mg. supplement produced even a less decisive result. The test rat gained the faster in four of the eight pairs and attained the greater body length in five of the pairs. While the average differences between pairs favored the sucrose supplement, they were smaller than in the first experiment, i.e., 0.50 gm. and 0.9 mm., respectively.

The 400-mg. supplement to the solid diet was clearly effective in promoting a more rapid growth. In seven of the eight pairs the rat consuming the added sucrose gained the faster and attained the greater body length. The average excess gain of the sucrose rats was 6.5 gm., the standard deviation of differences in gain was 5.87 gm., and the probability of a fortuitous outcome only 0.011. The mean excess body length of the sucrose rat was 2.5 mm., the standard deviation 2.5 mm., and the desired probability only 0.017.

The results of adding a daily supplement of 400 mg. of sucrose to the basal diet of mineralized milk were equally decisive. The mean difference in body weight gain favoring the test treatment was 6.37 gm., the standard deviation of differences, 7.76 gm., and the probability of a fortuitous outcome, 0.034. With reference to the differences in final body length, the statistical results are:  $M=2.62$  mm.,  $s=2.45$  mm., and  $P=0.013$ .

In this latter test the rats were destroyed and the heats of combustion of their empty carcasses determined. Comparing the energy contents of pair mates, the mean difference was 18.25 calories favoring the test treatment, the standard deviation of differences was 16.88 calories, and the probability of a chance outcome only 0.012.

A reasonable interpretation of these auxiliary experiments is that, in a paired-feeding test involving eight pairs of rats and continuing for 6 weeks, the biological error (measured by the standard deviation of differences in performance between pair mates) is such as to obscure completely the nutritive effect of a daily supplement of 100 and of 200 mg. of sucrose, while it is not sufficient to obscure completely the nutritive effect of 400 mg. of sucrose. It is fair to conclude that the biological error is just large enough to completely obscure the effect of about 300 mg. of sucrose consumed daily. This line of reasoning leads to the expectation that, with a larger number of pairs of experimental animals (and also after a longer feeding period), this obscuring effect of the biological error would diminish until with an infinite number of pairs it would disappear entirely.

If under the conditions of these experiments the nutritive effect of a daily supplement of 300 mg. of sucrose, containing 1.18 calories, is inappreciable, it seems reasonable to assume that the observed nutritive effect of a larger supplement,  $a$  grams, containing  $b$  calories, would actually be the effect of  $a-0.3$  gm. of sucrose, or of  $b-1.18$  calories from sucrose. Hence, the percentage recoveries of the sucrose calories given in the last column of table 6, may be recomputed by dividing

the total calories accounted for, given in the next to the last column, by  $357 - (1.18 \times 36) = 315$  calories, there being 36 days in the experiment. The recomputed percentages average 56, as compared with the uncorrected average of 49.

In making a similar correction of the recovery percentages for alcohol, there is a complication in the fact that the energy of alcohol appears to be less available for physiological purposes than the energy of sucrose, basing this judgment on the average percentage recoveries of 34 for alcohol and 49 for sucrose. If the nutritive effect of a daily supplement of 1.18 calories as sucrose is just obscured by the biological error of the method, it may be expected that a larger calorie supplement in alcohol would exert no appreciable effect. Assessing the daily supplement at  $1.18 \times \frac{49}{34}$  and deducting this value, multiplied by 42 (the length of the experiment in days), from the total intake of alcohol calories (table 5, column 2), permits the calculation of first approximations to the correct percentage recoveries. These approximations average 44. A second approximation is obtained by the use of a daily factor of  $1.18 \times \frac{56}{44}$ , instead of  $1.18 \times \frac{49}{34}$ . These approximations average 42 per cent.

The specific dynamic effect of sucrose in the rat has not been determined, in so far as the writer is aware, but Kriss, Forbes and Miller ('34) have attempted to determine the heating effect of a starch supplement of 2.2 gm. added to a basal maintenance diet. This effect would presumably approximate the extra heat produced by a supplement of 2.5 gm. of sucrose added to a maintenance diet, but may be considerably less than the specific dynamic effect of the same supplement added to a growth-promoting diet (Forbes, Kriss and Miller, '34). However, in using the data of Kriss, Forbes and Miller, it is necessary to recompute the specific dynamic effects, since all heat production values have been reduced to a constant rat weight of 100 gm., using the ratio of the two-thirds powers of the actual and the standard weights. This so-called 'correction' involves the wholly gratuitous assumption that the specific dynamic effect of a given amount of a nutrient when

fed to rats of different weights varies in direct proportion to the surface areas, the larger rats exhibiting the greater effects. While little or no direct evidence on this point is available one might expect a greater, rather than a smaller, heating effect of a given supplement with the smaller of two rats, because it would represent a higher plane of nutrition (Forbes, Kriss and Miller, '34). In the experiments of Kriss, Forbes and Miller the rats were all lighter when tested with the maintenance ration than when tested with the maintenance ration to which the starch supplement was added, the former weights averaging 100 gm. and the latter 122 gm. The recomputed specific dynamic effect, based upon the assumption that only the basal heat varies with the body surface,<sup>2</sup> the heat increment being unaffected by size of rat, is equivalent to 31.5 per cent of the energy of the starch.<sup>3</sup>

If one may assume on the basis of these results<sup>4</sup> that 2.5 gm. of sucrose added to the maintenance diet of a rat will increase the heat production by an amount equivalent to 31.5 per cent of its energy content, one would expect, on the basis of other

<sup>2</sup> This assumption is used in all of the recent calculations of heat increments of rations for steers by Forbes and his associates. No reason is given for departing from this practice in the rat experiments.

<sup>3</sup> Incidentally when all of the results in this article are recomputed as indicated, the specific dynamic effects of the nutrients studied become:

	<i>Added to a maintenance diet, per cent</i>	<i>Fed exclusively,<sup>1</sup> per cent</i>
Casein	38.2	30.3
Starch	31.5	20.8
Olive oil	26.2	14.0

<sup>1</sup> Corrected for sparing effect on body protein and fat.

The lack of similarity between these two series of values offers no support for the theory that body nutrients undergoing catabolism exert a specific dynamic effect.

<sup>4</sup> Some hesitation is felt in using even this recomputed specific dynamic effect, because the heat production in all experiments is assumed to be constant throughout the 24 hours. No adequate justification is offered for this practice, concerning which considerable scepticism may reasonably be felt. The average hourly heat production for the day is probably less than the heat production of the 4- or 5-hour experimental periods. It is also possible that the size of the specific dynamic effect is dependent upon the composition of the basal diet.

results from the same laboratory (Forbes, Kriss and Miller, '34), that the same supplement added to a super-maintenance diet may exert even a greater heating effect, since the curve relating heat production and food intake in rats continues a sharp upward turn at levels above the maintenance.

The average disposition within the body of the energy in the sucrose supplements may be pictured as follows. Of the total intake of 357 calories, the effect of 42 calories (12 per cent) was completely obscured by the biological error of the method, the 'extinction quota,' if we may call it such, being equal to 1.18 calories daily. Of the 315 physiologically effective calories, 99 calories (32 per cent) were stored as the new tissues of growth, 62 calories (20 per cent) were expended on the increased basal functions incident to the increased size of body, 15 calories (5 per cent) were, directly or indirectly, represented in the excretion of metabolic fecal products, and 99 calories (31 per cent), or more, were dissipated as heat, constituting the specific dynamic effect. The remaining 40 calories (12 per cent) may have participated in the specific dynamic effect, or they may have been lost in the increased energy expenditure in muscular activity incident to the increased body size.

The average disposition of the alcohol calories followed a somewhat different plan. An average of 307 calories from alcohol were consumed per rat in a total of 42 days. The physiologically ineffective calories (the 'extinction quota') were estimated at 1.59 calories daily, a total of 66 calories (21 per cent). Of the remaining 241 calories, 67 calories (28 per cent) were deposited as the new tissues of growth, 42 calories (17 per cent) were spent on the increment in basal functions consequent upon increased body size, and 6 calories (2 per cent)<sup>5</sup> were gained to the body by the increased digestibility of the basal ration occasioned by the alcohol supplements. The remainder of 138 calories (57 per cent) was probably mainly dissipated as heat.

<sup>5</sup> These 6 calories may be considered as contributing to the energy balance of 67 calories, of which the alcohol can be credited with only 61.

## CONCLUSIONS

The energy of ethyl alcohol is to a large extent available for physiological purposes. Added to a complete diet, such as milk, it induces more rapid growth and a greater retention of nitrogen as well as of fat. The new tissue traceable to the alcohol supplement possesses a greater content of fat than that produced on the basal diet alone. The alcohol supplement does not increase the excretion of metabolic products in the feces, but probably does increase the digestibility of the basal diet.

As compared with a similar supplement of sugar, the energy of an alcohol supplement is only about three-fourths as available for physiological purposes, probably because of a greater specific dynamic effect. Its growth-promoting power is definitely less, though the composition of the new tissues produced is similar to that of the gains in weight produced by a sucrose supplement. Its effect on digestion is quite different from that of a sucrose supplement, the latter exerting no appreciable effect on the digestibility of the basal diet, but inducing a greater excretion of metabolic products in the feces in accordance with its content of dry matter.

The biological error in paired-feeding experiments involving eight pairs of rats and continuing for 6 weeks is such that the nutritive effects of a daily supplement of about 1 calorie in the form of sucrose, added to the diet of one rat in each pair, is completely obscured.



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# A STUDY OF IRON METABOLISM WITH PRESCHOOL CHILDREN <sup>1</sup>

LEAH ASCHAM

*Department of Home Economics, Georgia Experiment Station, Experiment*

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Several years ago a dietary regimen was instituted in the preschool cottage of a home for dependent children as a long time feeding experiment. After the adequate diet had been in use for some time an iron utilization study was initiated since data concerning the iron requirements of young children were limited. The results of three investigations have now appeared in the literature. Rose et al. ('30) and Daniels and Wright ('34) propose the equivalent of 0.60 mg. per kilogram of body weight as being adequate to meet the needs of maintenance and growth of children in the preschool group while Leichsenring and Flor ('32) state that 0.48 mg. will provide for a 50 per cent margin of safety. The data reported herein more nearly agree with the higher figure.

## EXPERIMENTAL PROCEDURE

Six normal children, four boys and two girls, whose complete cooperation seemed assured, served as subjects for the experiment. Their ages ranged from 4 to 6 years and their weights from 17.1 to 19.3 kg. During the entire experimental period of 19 consecutive days the established schedule of the home was followed in so far as the strict supervision, necessitated by the nature of the study, permitted. This included regularity of meals and in hours of sleep, play out-of-doors, and light chores assigned to the several children. Urine and

<sup>1</sup> Published with the permission of the director as journal series no. 41, Georgia Experiment Station.

feces, marked with carmine, were collected quantitatively for five periods of 3 days each.

Table 1 lists the weights and average iron content of the different foods served daily.

The children, without exception, consumed all of the food served at each meal and only two adjustments had to be made during the entire time. The portion given each person was the same from day to day with one modification. Bread was the only article in the diet which was designed to fluctuate with the individual desire for bulk and consequently differed

TABLE 1  
*Average daily intake of iron*

FOOD	WEIGHT	AVERAGE IRON CONTENT
	<i>gm.</i>	<i>mg.</i>
Cream of Wheat	20	0.34
Rice	25	0.12
Bread	90	1.44
Potatoes	100	0.80
Milk	600	0.58
Cottage cheese	30	0.11
Butter	33	....
Cabbage	30	0.08
Tomatoes (canned)	75	1.09
Turnip greens	200	3.24
Apples (canned)	75	0.58
Pineapple (canned)	50 (slice)	2.44
	15 (juice)	

with each child, and also varied somewhat daily. Furthermore, it seemed advisable after the eighth experimental day to slightly reduce the amount of turnip greens in order to insure continued success in complete intake of food.

The food for each child was cooked separately in pyrex or new enamel utensils. Butter was the only article which was weighed for a 3-day period, all other supplies were checked daily. An adequate quantity of rice and Cream of Wheat was provided for the entire experiment while a sufficient amount of apples, tomatoes, pineapple and cottage cheese was prepared for each 3-day period. Potatoes were selected from

the same bin. Milk for cooking and drinking was obtained from the home's dairy and turnip greens were picked each morning from special plantings. Bread was delivered daily and cabbage was purchased every third day. Distilled water was used in the preparation of food and by the children *ad libitum*.

Food for iron analyses was procured as follows:

a. One sample of Cream of Wheat, rice and potatoes representing the entire time.

b. One sample of apples, pineapple, tomatoes, and cheese (two in this instance) made up of aliquots from each period.

c. A composite of daily aliquots of bread, milk, turnip greens, and cabbage for each of the five experimental periods.

Feces and urine were collected by periods. All foods and excreta were kept in glass containers until ready for drying and analysis.

The method for the determination of iron with the details of the technique has been published elsewhere (Ascham, '31).

#### DISCUSSION

In choosing the diet for this experiment foods low in iron, as shown in the tables of Peterson and Elvehjem ('28), were selected. Except for the iron in canned pineapple which was higher than estimated and that in turnip greens which was lower, the calculated and actual intakes differed by less than 1 mg. for a 3-day period.

No data for the iron content of urine are given. It was concluded, after several analyses, that the influence of the urinary iron upon the final balance would be comparatively insignificant since it was low and relatively constant. Daniels and Wright found 94 to 97 per cent of the iron excretion in the feces, and Rose and co-workers over 95 per cent. Similar low iron output in the urine was reported by Leichsenring and Flor though in their subjects it represented a much higher percentage of the total excretion. According to Marlow and Taylor ('34), the daily urinary iron is also constant in normal adults.

Retentions by periods for two children are given in table 2 and are representative of the group as a whole.

As shown in table 2, H.E. maintained positive balances throughout. During period 4, E.W. had a large negative balance, the largest of any child during the entire experiment; he also had the widest range of retention. Another subject, J.E. maintained a positive balance in all periods, but the remaining three children had two negative balances each. During period 2, D.F. reached a positive balance of 14.39, the largest during the experiment. In only one case, that of E.W., did the highest retention occur in the first period. The four subjects not given in table 2 had the following balances during the first 3 days, —1.38, —1.36, —6.07, and +3.43.

TABLE 2  
*Intake, output, and balance of iron by periods*

EXPERIMENTAL PERIOD	H. E.			E. W.		
	Intake	Output	Balance	Intake	Output	Balance
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	30.20	22.37	+ 7.83	28.28	16.27	+ 12.01
2	34.48	33.92	+ 0.56	32.09	32.89	— 0.80
3	31.60	21.76	+ 9.84	30.43	24.66	+ 5.77
4	33.07	29.89	+ 3.18	31.34	41.81	— 10.47
5	31.24	24.07	+ 7.17	29.73	22.42	+ 7.36

Their respective highest balances were +14.39, +7.14, +10.20, and +11.49 which occurred in periods 2, 3, 5, and 3, respectively. Just previous to the first experimental period the medical charts registered hemoglobins of 75 to 82 per cent. It would seem, therefore, that the children had been in fairly good nutritional condition previous to this metabolism study.

The type and number of stools varied with different individuals. There were never less than two passages a day, three or even four were not infrequent, and several times quite soft feces were encountered.

Even with some negative balances, positive retentions were maintained for each subject for the five periods as shown in

table 3 which gives the daily average of the 15 days. It is possible that one or two might have become negative if the urine figures had been included.

The iron requirement as indicated in this study is in fair agreement with the standard of 0.60 mg. per kilogram proposed by Daniels and Wright and by Rose et al. but higher than the allowance of Leichsenring and Flor. The frequent, loose stools, without doubt, had some influence upon the output. Further, it is possible that some of the differences in retention reported by the different investigators might be explained upon the basis of available iron since the diets

TABLE 3  
*Average daily iron balances for six preschool children*

SUBJECT	SEX	WEIGHT	TOTAL IRON			IRON PER KILOGRAM BODY WEIGHT		
			Intake	Output	Balance	Intake	Output	Balance
		<i>kg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
D.F.	Female	18.5	10.56	9.11	1.45	0.57	0.49	0.08
H.E.	Female	19.3	10.71	8.80	1.91	0.55	0.46	0.10
E.L.	Male	17.3	10.91	10.38	0.52	0.63	0.60	0.03
W.H.	Male	19.0	10.74	10.58	0.16	0.57	0.56	0.01
E.W.	Male	17.7	10.13	9.20	0.92	0.57	0.52	0.05
J.E.	Male	17.1	10.98	8.36	2.62	0.64	0.49	0.15

varied widely. Elvehjem, Hart, and Sherman ('33) and Sherman, Elvehjem and Hart ('34 a, '34 b) found the availability of the iron in the materials which they tested ranging from 25 to 100 per cent.

#### SUMMARY

An iron balance study was conducted for 15 days with six normal children, ages ranging from 4 to 6 years and weights from 17.1 to 19.3 kg.

Iron retentions varied from 0.01 to 0.15 mg. per kilogram with intakes of 0.55 to 0.64 mg. per kilogram, averages 0.07 and 0.59 mg., respectively.

It is assumed that the availability of iron in foods may, in part, account for some of the differences found in the various reports upon the utilization of this mineral by young children.



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# RAT'S MILK AND THE STOMACH CONTENTS OF SUCKLING RATS <sup>1</sup>

D. T. MAYER

*Laboratory of Biological Chemistry, University of Missouri School of Medicine,  
Columbia*

(Received for publication April 26, 1935)

## INTRODUCTION

In spite of the recognized importance of the role played by the rat as a laboratory animal in nutritional investigation, practically nothing can be found in the literature concerning the character of the diet received by the suckling rat. Some years ago a very brief account of an analysis of the stomach contents of suckling rats was made in a Japanese scientific journal. Our recent efforts to locate this paper have been in vain. Pizzi (1894) included a reference to rat's milk in a paper upon milk of different species of animals. Other than this, we find no indication that the diet of suckling rats has been studied.

Efforts were first directed toward securing rat's milk from the mammary glands of the lactating female rat. However, the arrangement of the mammary glands in a flat, diffuse form makes the securing of the milk a difficult problem, since it prohibits the use of miniature pumps modeled after the human breast pump.

Our next efforts were directed toward a complete analysis of the stomach contents of suckling rats and a study of the conditions existing in their stomachs.

<sup>1</sup> This work has been previously presented in partial fulfillment of the requirements for the degree of master of arts at the University of Missouri.

## EXPERIMENTAL

The young rats chosen to supply the milk curd from their stomachs were from 10 to 18 days of age. The latter is about the maximum age at which it is normal to obtain a curd uncontaminated with other food.

The young rats were removed from the cage containing their mother rat at 9 P.M. on the evening before they were to be slaughtered, and kept over night without food or water. At 9 A.M. the next morning they were allowed to suckle as long as they would do so, and then immediately removed, killed by anesthetizing with ether in a closed chamber, and the stomachs dissected out.

An incision was next made in the wall of the stomach from which the contents were allowed to fall into a weighed bottle fitted with a ground glass stopper. Suitable samples were withdrawn from the weighing bottle for the various analyses after the contents had been weighed and thoroughly mixed.

Representative samples of approximately 3 gm. each were withdrawn for the moisture determinations. Drying in an electric oven at atmospheric pressure and a temperature of 90°C. as well as drying in a vacuum oven at 120°C. proved inadvisable since discoloration occurred with loss of water from the sugars in the material. A sample of material dried for 48 hours at 90°C. gave no qualitative test for sugar when tested with Benedict's reagent. Hence it was thought best to place the sample in a vacuum desiccator, then generate the highest vacuum possible with a Cenco oil pump, and allow the sample to dry in this manner until no further change of weight occurred.

Either the Kjeldahl-Gunning or the micro-Kjeldahl method of Cavett ('31) was used for the total nitrogen determinations.

Most of the micro-chemical sugar methods were designed by their authors to apply to glucose. It was found necessary to compare and modify these methods in order to fit them for the determination of lactose. In general, the sugar was determined by agitating a weighed sample of the stomach

contents in 5 to 10 cc. of saturated benzoic acid solution to break the curds, precipitating the proteins by a suitable method, filtering, and then diluting the filtrate to a suitable volume. Portions of the diluted filtrate were then analyzed.

Preliminary tests indicated that although the solutions were already casein-free, the protein precipitation was indispensable. It is a well-known fact that in sugar determinations the pH of the solution is important. Most of the standard methods of protein precipitation gave a more acid filtrate than the standard sugar solution against which it was to be compared. At first these acid filtrates were carefully neutralized, but in the later analyses the proteins were removed by the iron method of Steiner, Urban and West ('32), which gave a neutral filtrate.

A known solution of lactose was hydrolyzed and the resulting sugars determined by the revised Folin-Wu ('29) and the Folin-Malmros ('29) technic. The theoretical amount of galactose and glucose in the hydrolysate was found by both methods when the acid present was neutralized, but errors up to 30 per cent were introduced when no neutralization was made. The above results and the results obtainable by the Folin-Wu method upon cow's milk led us to use it in the majority of the sugar analyses.

The fat was determined by first drying 2- to 3-gm. samples of the stomach contents in vacuo, then extracting for 12 hours with anhydrous ethyl-ether in a Soxhlet extraction apparatus. The ether was removed from the fat by distillation and the fat weighed.

The saponification number of the fat was also determined.

The method of Van Slyke ('23) for chlorides in blood and tissues was adaptable for the determination of chlorides in 1- to 2-gm. portions of the stomach contents.

The pH and titratable acidity were investigated in various samples of the stomach contents.

## RESULTS

*Weights of the stomach contents*

A characteristic variation in the weights of the stomach contents was noted. A steady increase in stomach content weight up to the twelfth day was followed by a more rapid increase in weight for the next 7 days. At the seventh day the average weight of the contents was but 0.197 gm., at the twelfth day they weighed 0.8086, and at the nineteenth day the weight had increased to 1.5903 gm.

TABLE 1  
*Protein analyses*

SAMPLE NUMBER	SOLUBLE NON-PROTEIN NITROGEN, PER CENT		SOLUBLE PROTEIN NITROGEN, PER CENT		SOLUBLE PROTEIN, PER CENT		TOTAL, NITROGEN, PER CENT		TOTAL PROTEIN PER CENT WET BASIS
	Wet basis	Dry basis	Wet basis	Dry basis	Wet basis	Dry basis	Wet basis	Dry basis	
I Dry weight = 22.2 per cent	0.4406	1.99	0.4664	2.10	2.99	13.4	1.51	6.81	6.89
II Dry weight = 22.2 per cent	0.437	1.97	0.470	2.12	3.00	13.5	1.509	6.80	6.89
III Dry weight = 22.2 per cent	0.438	1.98	0.469	2.11	3.00	13.5	....	....	....

*Appearance of the stomachs*

The appearance of the stomachs proved to have an important bearing upon the desirability of the contents for the analyses. Usually the stomachs were fully distended. When newly filled, they had a milk-white appearance. At later stages the digested portions were an amber color and were usually found near the pyloric end of the stomach, where the digestion unquestionably first began. If food, other than milk had been ingested, it could easily be detected. In most cases the curds were well-formed. Occasionally, however, curd

TABLE 2  
*Sugar and fat analyses*

SUGAR			FAT			
Sugar method used	Per cent lactose in sample	Method of protein precipitation	Fat per cent		Saponification number of fat	Molecular weight calculated from average saponification number
			Wet basis	Dry basis		
1. Folin-Malmros neutralized	3.41	H <sub>2</sub> SO <sub>4</sub> -Na-tungstate	12.4	55.8	208.6	801.1
2. Folin-Malmros neutralized	3.36	Hcl-tungstic acid (7 per cent Hcl)	12.4	55.8	211.6	
3. Folin-Malmros neutralized	3.38	Hcl-tungstic acid (7 per cent Hcl)	12.4	55.8	210.8	
4. Folin-Wu	3.37	Folin's blood method	12.4	55.8	210.6	
5. Folin-Wu	3.37	F <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> -BaCO <sub>3</sub>	12.4	55.8	210.6 (average)	
6. Folin-Wu	3.39	F <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> -BaCO <sub>3</sub>	....	....	....	
7. Folin-Malmros	3.42	F <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> -BaCO <sub>3</sub>	....	....	....	

<sup>1</sup> Moisture of all samples = 77.8 per cent, dry weight = 22.2 per cent.

TABLE 3

SAMPLE AND DRY WEIGHT	TOTAL CHLORIDES AS Nacl PER CENT		TOTAL CHLORIDES AS Hcl PER CENT		TITRATABLE ACIDITY CUBIC CENTIMETERS OF N/10 NaOH PER 100 GM. OF CONTENTS	ACIDITY EXPRESSED AS PER CENT BY WEIGHT OF Hcl IN CONTENTS		HYDROGEN ION CONCENTRATION EXPRESSED AS pH
	Wet basis	Dry basis	Wet basis	Dry basis		Wet basis	Dry basis	
22.2	0.234	0.778	0.146	0.498	83.9	0.306	0.908	5.8
22.2	0.234	0.779	0.146	0.498	83.85	0.30	0.90	
22.2	0.234	0.779	0.146	0.498	83.9	0.306	0.908	

TABLE 4

*Complete analyses of the sample of stomach contents which most closely approached normal rat's milk as indicated by lack of curd formation*

MOISTURE, PER CENT	PROTEIN, PER CENT	CARBOHYDRATE (SUGAR), PER CENT	FAT, PER CENT	CHLORIDES (As Hcl), PER CENT
77.84	6.89	3.36-3.42	12.4	0.146

formation had progressed but little, so that the contents were semi-fluid in character. The semi-fluid contents were the most desirable for analyses.

### *Dry weight determinations*

The dry weight of the contents varied from 22.2 per cent to 45.3 per cent, depending upon the time between suckling and the securing of the samples. Samples secured immediately after suckling were semi-fluid, hence were more representative of a normal milk. The semi-fluid contents had a moisture of 77.8 per cent or a dry weight of 22.2 per cent.

Evidence, as yet not fully substantiated, is indicative of a low tryptophane-containing casein for rat's milk.

### DISCUSSION

The percentage of solids present in the stomach contents was dependent upon the time that the milk remained in the stomachs—the moisture decreasing as the time increased. As the curd formed, fluid was expressed from the curdling mass and it soon passed out of the stomach. Before this process the percentage of dry weight is of a similar order of magnitude to various other milks although greater than the average of milks that have been reported upon.

The very slight variation in the percentage of fat calculated upon the basis of the dry weight indicates the lack of a significant quantitative change in the fat while in the stomach.

The sugar, chlorides, and soluble protein are dependent upon the quantity of water present. Since they are all soluble in water, they are undoubtedly removed in the fluid expressed from the curd and passed with the fluid into the intestines. The hydrochloric acid of the gastric juices increases the total chloride values.

The proteins of the stomach contents might vary slightly. The digestive juices may add varying quantities of protein and a slight additional variation through protein digestion is possible. But as already stated, digestion is easily detected by a color change in the contents from white to amber, and

analyses of partially digested samples were excluded from the tables.

In spite of the possible slight variations in the analyses of the stomach contents, these analyses do give relative values; and, in most cases, these values are a fair indication of what one should expect for the normal milk.

Those analyses which most nearly represent the original, unmodified milk, indicate about 22.2 per cent solids, of which the average values are: for fat 55.8 per cent, for proteins 31.03 per cent, and for carbohydrates 15 per cent.

Three milks—cow's, goat's and human—are widely used in human nutrition, especially infant nutrition. From the standpoint of nutritional interest a comparison of these milks with rat's milk should be of value.

Rat's milk contains six times the protein of human milk and about three times that of either cow's or goat's milk.

The fat of rat's milk is about four times that of either human or cow's milk. Animals mentioned in the literature as having a milk of exceptionally high fat content are large in most cases. It is a notable fact that large ocean-living mammals have a very high percentage of fat in their milks, especially the dolphin with 43.46 per cent, Hammarsten ('10). Of the smaller animals mentioned in the literature, the milks of the dog, cat and rabbit, Folin, et al. ('19), show the highest percentage of fat.

#### SUMMARY

The analyses reported of protein, fat and sugar indicate a high fat and a low carbohydrate diet for the suckling rat.

Fats comprise approximately 55 per cent of the solids in rat's milk, approximately 30 per cent are proteins, and approximately 15 per cent are carbohydrate.

Calculating the proportion of calories upon the basis that fat yields 9.3, protein about 4, and disaccharides 3.95 calories per gram, 74 per cent of the calories in the diet of the suckling rat are furnished by fat, 18 per cent by protein, and only 8 per cent by carbohydrate.



In spite of the great proportion of fat in the diet of the suckling rat, this diet is not an extremely ketogenic one. The ketogenic-antiketogenic ratio, calculated as suggested by Shaffer ('21 a, '21 b, '22), is 1.35:1.

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# SPECTRUM ANALYSIS OF HEN EGGS AND CHICK TISSUES

W. F. DREA

*Laboratory of the Colorado Foundation for Research in Tuberculosis,  
Colorado College, Colorado Springs*

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There is an increasing amount of evidence for the biological importance of certain 'trace' elements.

The investigation of hen eggs and chick tissues was made to determine what elements passed from the rations and drinking water into the blood of the laying hen, hen eggs and tissues of chicks which had not ingested food or water.

The eggs and chicks were from a group of eggs laid at the time the water and rations investigated were being ingested by the laying hens. The blood of one laying hen was also secured at this time.

## MATERIAL AND METHOD OF INVESTIGATION

The water was evaporated in silica and platinum dishes. The residue was used for analysis as there was very little organic material present. Ashing was not necessary.

Mash and a combination of wheat and corn were being fed to the hens at the time of investigation. The two kinds of feed and the yolks, whites and shells of five eggs were each ashed separately. This was done at low red heat in silica dishes. Each ash was separately analyzed. The blood of one of the laying hens was subjected to a similar procedure.

Three weeks later, ten chicks hatched in an incubator were used for the investigation of some of their tissues. The chicks had not ingested water or food. About 48 hours after being hatched, they were made unconscious by a sharp blow over

the top of the head. Sufficient feathers were removed from around the necks and the heads cut off. A few drops of the blood were allowed to escape, and the remainder was received in a platinum dish. The gizzard, kidney, brain, eye, lung, thigh muscle, liver, heart, blood and femur were separately ashed. The same method was used as for the eggs and feed. Most of the organs such as the kidneys, were so small that they had to be pooled. The blood of the chicks was also pooled. In addition, a femur from a 96-hour chick that had not ingested water or food was ashed.

No effort was made to burn off all the carbon in the substances which were resistant, and no reagents were added to promote ashing.

Precautions were taken to avoid contamination by extraneous elements. Spectra of the electrodes used were secured to note the presence of any elements already in them. The shell of an egg taken from the oviduct of a hen was analyzed as a control to eliminate the question of possible contamination of the laid eggs. The ashing and water evaporation was done in a room free from air currents, where presumably the dust in the air had previously settled.

The technic of securing the spectra was in general as reported before by Drea ('34). A direct current of 7.5 amperes was used for excitation of the spectrum after 10 to 20 mg. of the ash were placed on top of the lower of the two Acheson graphite spectroscopic electrodes. Iron arc spectra were used for measuring the wave lengths of the most 'persistent lines' of the elements as given in the International Critical Tables for arc spectra. Not all of the elements can have their spectra excited by the arc. Fluorine was sought by means of the  $\text{CaF}_2$  band. A Hilger medium-sized quartz spectrograph was used. Eastman type III-F and type IV-F spectroscopic plates had the spectra recorded upon them. At least three spectra were photographed for each substance investigated.

No attempt at quantitative determinations was made.

## RESULTS

Elements are reported as present or absent according to whether or not their characteristic wave lengths are present in the spectra of the investigated substances. Elements reported as absent may have been present in too small quantities to be revealed with the technic employed although generally speaking the spectrographic method is extremely delicate.

## DISCUSSION AND SUMMARY

A number of elements pass from the feed and/or water into the hen's blood, from there into the egg, and finally into all of the chick's tissues and chick's blood. These are aluminum, barium, calcium, copper, iron, magnesium, phosphorus, potassium, rubidium (?) silicon, sodium, strontium, titanium and vanadium. Manganese and zinc were each absent from one organ and the chick's blood. Of the 'trace' elements, aluminum, barium, copper, silicon, strontium, titanium and zinc were quite uniformly distributed among the tissues.

There is a group of 'trace' elements more concentrated in the hen's blood and/or egg than in the feed and it appears reasonable that these are of high physiological importance. These are barium, iron, strontium and vanadium. The importance of iron is of course well known. The selection of barium and strontium may be caused by the inability of the hen's organism to distinguish these elements from calcium. The selection of vanadium is more difficult to explain: it is present in 4 plus quantities in the blood of both chick and hen, in the femur, heart, kidney and lung, suggesting some function associated with the hematopoietic organs.

There is a group of three elements whose presence in the newly hatched chick is unnecessary or harmful. These are boron, fluorine and silver. Boron although present in the food and water was found only in the egg yolk. Fluorine was present in the water in a concentration of about 2 p.p.m. It was not present in a sufficiently large amount to be demonstrated in the eggs, bloods or soft tissues. It was present in

*Trace elements*

	DRINKING WATER	BATONS	HEN'S BLOOD	YOLKS	WHITES	SHELLS	CHICK'S BLOOD	BRAIN	EYE	FEMUR 48-HOUR	FEMUR 96-HOUR	GIZZARD	HEART	KIDNEY	LIVER	LUNG	MUSCLE
Al	+++	++	++	++	++	+	++	+	++	++	++	++	++	++	++	++	++
Ba	++	++	++	+++	+++	+++	++	++	++	++	++	++	+	++	++	++	+
B	+	+	—	+	—	—	—	—	—	—	—	—	—	—	—	—	—
Cr	+	+	+	3 out of 5 +	2 out of 5 +	1 out of 5 +	++	++	++	++	++	++	++	++	++	++	++
Cu	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
F	+++	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Fe	++	++	+++	+++	+	+	+++	+	+	+	+	++	+++	+++	+++	+++	+++
Pb	+	++	+	4 out of 5 +	2 out of 5 +	+	+	+	—	—	+	++	—	++	++	++	+
Mn	++	+++	+	+++	—	—	—	++	—	—	—	++	++	++	++	++	—
Mo	+	+	+	1 out of 5 +	+	4 out of 5 +	—	+	+	+	+	+	+	+	+	+	+
Rb	—	++	++	+	1 out of 5 +	—	++	+	?	?	?	+	+	+	+	+	++
Si	+++	+	+	+++	++	++	+	+	+	+	+	+	++	++	+	+	+
Ag	+	—	—	—	3 out of 5 +	2 out of 5 +	—	—	—	—	+	—	—	—	—	—	—
Sr	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Ti	++	+	++	++	+	+	+	++	+	+	+	+	+	+	+	+	+
V	+	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Zn	—	+	+	++	—	3 out of 5 +	—	+	+	+	+	+	+	+	+	+	—

Larger amounts of elements Ca, Mg, P, K and Na present in the usual larger amounts for such organic substances.

one femur and therefore must have been present in the egg from which this femur was evolved. This element is most probably of no physiological importance in any amount and when present in drinking water to the amount of more than 1 p.m.m. has a toxic effect upon the developing enamel of children's teeth. Silver was present in the water. It was not demonstrated in the hen's blood, egg yolk, chick blood or tissues with the exception of one femur.

Chromium, lead and molybdenum were present in approximately equal amounts in the hen's blood and in the feed and water, but were not constantly present in the eggs. Although the hen tolerates these elements in her own blood stream, she attempts to reject them for the formation of her eggs. The presence of these elements is tolerated in the adult organism but their presence is not necessary for the functioning of the chick and may be harmful. When the chicks contained these elements, molybdenum was found principally in the liver, lead in the lungs and chromium in the brain and eye. While we expect the accumulation of harmful heavy metals in the liver, the accumulation of chromium in the brain, and lead in the lung is surprising and at present unexplained.

Manganese was present in greater quantity in the yolks as compared with the hen's blood and was absent from the egg white and egg shell. It was concentrated in the liver and appeared in slightly lesser amounts in the kidney and gizzard. Its absence from the chick's blood and its presence in all of the tissues with the exception of the eye indicates a probable inadequate supply of this element which is of general nutritive importance.

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# THE BASAL HEAT PRODUCTION OF THE RHESUS MONKEY (*MACACA MULATTA*)

NATHAN RAKIETEN<sup>1</sup>

*Laboratory of Physiology, Yale University School of Medicine, New Haven*

(Received for publication May 29, 1935)

The gaseous metabolism of the primates has only recently been the subject of investigation. Botschkareff ('33) determined the heat production of one macaque and three mandrills, but he was unable to secure basal conditions during observations. The only other published data are those of Bruhn ('34), who studied the basal metabolic rate of various species of primates including in his series four normal adolescent rhesus monkeys (*Macaca mulatta*). In view of the paucity of information concerning the metabolism of the rhesus monkey it has seemed worth while to record further studies on the basal heat production in this form.

## METHODS

Six female and five male monkeys were studied. They were normal adolescent animals the weights of which varied from 2.7 to 3.6 kilos. The animals were generally fasted 18 to 24 hours prior to observation, but in one series the post-absorptive period was lengthened to 36 hours.

The determinations were made in an open circuit system modelled after the apparatus devised at the Carnegie Nutrition Laboratory at Boston (Carpenter and Fox, '31). The animal, confined in a small cage, was placed in a chamber of appropriate size through which outdoor air was circulated by

<sup>1</sup>When this work was done the author was a Porter Fellow of the American Physiological Society. The research was aided by a grant from the research funds of the Yale University School of Medicine. The author is at present in the Physiology Department at Massachusetts State College, Amherst, Massachusetts.



a rotary blower. The outgoing air after passing through two dry gas meters was collected for analysis by the Fox bag method of aliquot sampling (Carpenter and Fox, '31). The samples so obtained were stored in Benedict ('27) respiration pumps, and then analyzed in the Carpenter ('33) gas analysis apparatus. An activity recorder modified from that described by Benedict and Homans ('12) was used to register the animal's movements. Usually aliquots from four to five 20-minute periods were collected and the results of the two periods which gave the best agreement were taken to denote the animal's heat production. The lowest values were chosen in every instance. An agreement of 6 per cent or less was usually obtained for successive periods. Following the experiences of Bruhn ('34) the determinations were made at night to facilitate basal conditions.

The apparatus was tested intermittently during the investigation by burning alcohol in the chamber and by analyses of outdoor air. The mean respiratory quotient for alcohol in this series was  $0.664 \pm 0.003$ , while the greatest variations from the theoretical respiratory quotient of 0.667 were 0.674 and 0.658. The average results for the outdoor air analyses were 0.032 for  $\text{CO}_2$  and 20.942 for  $\text{O}_2$ .

The surface area of the animals was calculated by the method of Lee and Fox ('33).

#### RESULTS AND DISCUSSION

The complete data obtained in these experiments are presented in table 1. It was found that the average heat production of the eleven animals was 608 Cal. per square meter per 24 hours. This is approximately 7 per cent lower than the average figures given by Bruhn ('34) for the four normal animals studied by him. Eight of the eleven animals had metabolic rates that ranged from 586 Cal. to 640 Cal. and the remaining three, numbers 30, 4 and 37, gave values, respectively, of 543, 543 and 709 Cal.

The wide divergences from the mean of these three animals were undoubtedly due to their distinctive metabolic rates.

Animal no. 37 which gave the highest value of the group was extremely excitable and it is conceivable that this high heat production may be accounted for by the emotional disturbances pointed out by Benedict ('35) and by the cardiac and respiratory factors which have recently been demonstrated by Carpenter et al. ('34) to produce a considerable augmentation in the O<sub>2</sub> consumption of normal individuals in the basal state. It is interesting to note that Bruhn ('34) obtained a value of 701 Cal. on one of the animals in his series.

TABLE 1  
*Results of metabolic rate determinations on eleven rhesus monkeys*

ANIMAL NO.	NUMBER OF HOURS POST ABS.	SEX	WEIGHT IN KILOS	O <sub>2</sub> LITERS/HR.	R. Q.	CALS./SQ. M./24 HR.	CALS./SQ. M./HR.	CALS./KILO/24 HR.
1	18	♀	3.7	1.570	0.753	640.0	26.7	48.7
2	18	♀	3.5	1.382	0.770	586.0	24.4	45.1
3	18	♀	3.6	1.506	0.745	628.0	26.1	48.5
30	24	♀	3.6	1.331	0.762	543.0	22.6	41.3
37	24	♀	3.5	1.680	0.745	709.0	29.5	54.4
24	36	♀	3.1	1.330	0.705	601.0	25.0	49.1
Average of females:						618.0		
4	18	♂	3.1	1.174	0.794	543.0	22.6	43.6
45	18	♂	2.9	1.248	0.742	595.0	24.8	48.9
46	18	♂	2.8	1.296	0.737	632.0	26.3	52.2
44	24	♂	2.7	1.206	0.724	600.0	25.0	50.5
23	24	♂	2.9	1.309	0.721	622.0	25.9	51.0
Average of males:						598.0		
Total average of entire group:						608.0		

Further evidence that this result is valid is supplied by two experiments in which the animal was given a subanesthetic dose of sodium amytal, 35 mg./kilo, a drug which has been demonstrated by many investigators (Anderson et al., '30; Deuel et al., '26; and Siebert and Thurston, '32), to produce little or no alteration of the basal metabolic rate even in anesthetic quantities. The findings in these experiments are presented in table 2. The average heat production in the two types of procedure was almost identical, the difference being approximately 1 Cal. Similarly the low metabolic rates of

animals nos. 30 and 4, both of which gave values of 543 Cal., must be attributed to their individual differences. It was surprising to find two animals of different weight and sex that had the same low basal metabolic rate. It is perhaps unwise to use the average metabolism of the group.

The trend of similar metabolic determination for the two sexes was noted throughout the investigation. The average heat production of the female group was 3.2 per cent higher than the male, 618 Cal. as compared with 598 Cal., a difference, however, which is well within the experimental error. Moreover, if the values obtained on animal no. 37, a female, are excluded the average results for the female group becomes 600 Cal., a value closely approximating the heat production of the males. It is possible that when more data on the heat

TABLE 2

*B. M. E. of monkey no. 37 which received 35 mg. amytal/kilo*

	NUMBER OF HOURS POST ABS.	SEX	WEIGHT IN KILOS	O <sub>2</sub> LITERS/ HR.	B. Q.	CALS./ SQ. M./ 24 HR.	CALS./ SQ. M./ HR.	CALS./ KILO/ 24 HR.
No amytal	24	♀	3.5	1.680	0.745	709.0	29.5	54.4
Amytal	24	♀	3.65	1.702	0.748	710.0	29.6	54.7

production of this animal have been accumulated similar sex differences such as those observed in the human by Benedict and Talbot ('21) will be evident.

*Respiratory quotients.* The respiratory quotients of the ten animals which were 18 to 24 hours post-absorptive ranged from 0.72 to 0.79. The quotients of seven of these animals fell within the limits of 0.74 and 0.77, while the three remaining animals nos. 4, 23 and 44, gave values of 0.79, 0.72 and 0.72, respectively. A series of determinations were carried out on animal no. 24 when it was 36 hours post-absorptive and its respiratory quotient at this time was 0.705. It would appear that the respiratory quotients of these animals decreases more rapidly than that of other mammals, a phenomenon that may possibly be associated with the monkey's high carbohydrate diet. The respiratory quotients obtained in this investigation

were slightly lower than those reported by Bruhn ('34), a difference that probably resulted from our use of a longer post-absorptive period.

#### SUMMARY

1. The average basal heat production of eleven adolescent rhesus monkeys was found to be 608 Cal./square meter/24 hours.

2. A similar heat production was observed for the males and females.

2. Two experiments on one animal which received 35 mg. of sodium amytal/kilo gave results almost identical with those obtained on it under normal basal conditions.

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# EFFECTS OF INCREASING THE CALCIUM CONTENT OF A DIET IN WHICH CALCIUM IS ONE OF THE LIMITING FACTORS

H. C. SHERMAN AND H. L. CAMPBELL

*Department of Chemistry, Columbia University, New York City*

TWO FIGURES

(Received for publication June 13, 1935)

It has been shown in previous papers (Sherman and Campbell, '24, '28, '30) that a diet adequate in the sense that it maintains normal growth and health with successful reproduction and lactation, generation after generation, may still be capable of improvement with resulting enhancement of nutritional well-being.

In the case of this kind which has been most extensively studied, the starting point was our diet A, a mixture of five-sixths ground whole wheat and one-sixth dried whole milk with sodium chloride and distilled water. At present writing (June, '35) our rat families are still thriving in the thirty-seventh generation on this diet. Notwithstanding this extraordinarily rigorous demonstration of the adequacy of diet A, it is not an optimal diet, for diet B (which differs from it only in containing a different quantitative proportion of the same articles of food) is clearly a better diet as shown by its influence upon growth and development, upon adult vitality, and upon length of life.

In chemical terms, diet B represents an enrichment of diet A in at least four factors: 1) protein (with presumably increased intake of histidine, lysine, tryptophane and perhaps other 'nutritionally essential' amino acids); 2) calcium; 3) vitamin A; 4) vitamin G (in the sense of the Bourquin-Sherman ('31) method) and perhaps the accompanying 'new

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factor' (Booher, Blodgett and Page, '34). As this 'new factor' is relatively abundant in both milk and wheat (as well as in yeast, even after autoclave treatment) it must be contained in fairly liberal amounts in both diets A and B and may or may not play a part in the nutritional superiority of the latter. We are now engaged in somewhat extensive experimentation designed to determine which substances (in the chemical sense) are the 'limiting factors' in diet A—present in this food mixture in sufficient amount to permit of its being adequate, but below the optimal concentration as shown by the fact that diet A does not induce and support quite so high a state of nutritional well-being as does diet B.

The present paper records the results of our experiments with calcium as one of these limiting factors in an adequate but not optimal diet.

#### EXPERIMENTAL

The experiments here reported compare results with parallel lots or family groups of rats fed respectively upon laboratory diet 16 (the same as diet A) and laboratory diet 162 which differs from diet 16 in containing enough added calcium carbonate to give it the same calcium content as our diet B.

The initial lots or family groups of experimental animals each consisted of two males and three females. Parallel lots, made up when the rats were 4 weeks of age, were carefully matched as to heredity and size, and were kept strictly parallel as to all other conditions, and until the natural deaths of all the experimental animals. Inasmuch as more than one generation may be required for the full effect of a difference of diet, we have included also in this comparison parallel lots of offspring of the original matched lots in the second, third and fourth generations, these being likewise kept in lots of two males and three females each. The total numbers included in the experiments reported were: fifty-four males, seventy-seven females on diet 16; forty-nine males, seventy-two females on diet 162.

Many different numerical findings in the records of the life histories of the animals combine to serve as criteria of nutritional well-being. For the sake of conciseness these may be grouped under the three headings of, 1) rate and efficiency of growth, 2) indications of adult vitality, and 3) length of life.

*Rate and efficiency of growth.* The general average differences in growth and resulting size are shown in figure 1.

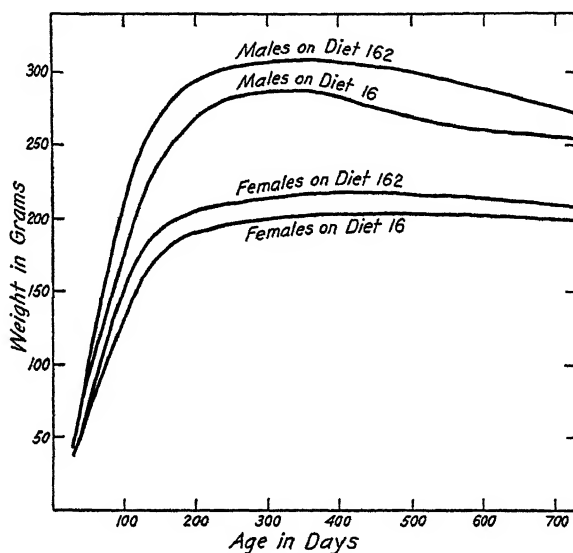


Figure 1

In each sex, growth was somewhat more rapid, and average size at a given age was somewhat greater, on the diet of more liberal calcium content (diet 162). Food consumption, per gram of body weight, was slightly higher on the same diet in the earlier stages of growth, but thereafter the food consumptions were essentially alike, so that in the general result the diet of higher calcium content was somewhat more efficiently utilized for growth and maintenance. The average ad libitum consumption of the two diets, in terms of calorie intake per 100 gm. of rat at different ages, is shown in figure 2.

A more precise quantitative comparison of the rate and efficiency of growth on the two diets during the fifth to eighth weeks, inclusive, of the lives of the rats is summarized in table 1.

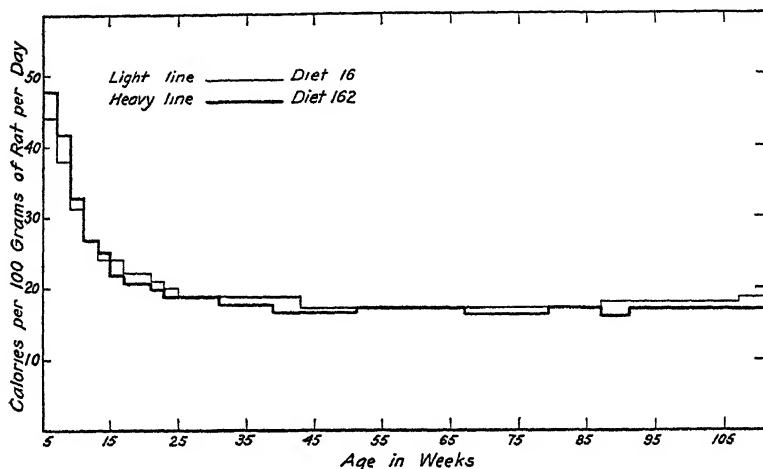


Figure 2

TABLE 1

*Influence of calcium intake on rate and efficiency of growth*

	DIET 16			DIET 162			DIFFERENCE WITH ITS PROBABLE ERROR
	Number of cases	C.V. <sup>1</sup>	Mean with its probable error	Number of cases	C.V. <sup>1</sup>	Mean with its probable error	
			<i>grams</i>			<i>grams</i>	
Gain in weight during 5th to 8th week, incl.							
Males	54	21.6	52.7 $\pm$ 1.01	49	23.0	67.8 $\pm$ 1.07	15.1 $\pm$ 1.47
Females	77	24.1	46.2 $\pm$ 0.82	72	18.5	59.0 $\pm$ 0.85	12.8 $\pm$ 1.18
Gain in weight per 1000 cal. consumed	131	13.3	64.9 $\pm$ 1.06	121	11.3	72.7 $\pm$ 1.00	7.8 $\pm$ 1.46
Gain in weight per gram of protein con- sumed	131	13.4	1.83 $\pm$ 0.030	121	11.5	2.06 $\pm$ 0.029	0.23 $\pm$ 0.041

<sup>1</sup> Coefficient of variation.

Thus growth during the period uncomplicated by puberty proves, on statistical analysis of the data from groups of from 49 to 131 cases, to have been significantly more rapid on the more liberal calcium intake and also significantly more efficient both on the basis of gain per 1000 cal. and on that of gain per gram of protein consumed. It should, however, be carefully noted that we are here dealing with only very moderate rates of growth, so that the higher of these seems well within the range of what is certainly advantageous. These experiments do not deal either with very rapid growth or with very

TABLE 2

*Influence of calcium intake on indications of adult vitality (breeding records of same females whose growth is shown in table 1 and fig. 1)*

	DIET 16		DIET 162		DIFFERENCE WITH ITS PROBABLE ERROR
	C.V.	Mean with its probable error	C.V.	Mean with its probable error	
Age at birth of first young, days	25.3	137.3 $\pm$ 2.79	22.9	115.9 $\pm$ 2.15	21.4 $\pm$ 3.52
Duration of ability to reproduce, days	65.6	184.9 $\pm$ 9.24	57.2	235.3 $\pm$ 10.56	50.4 $\pm$ 14.0
Number of young born	68.8	20.7 $\pm$ 1.10	63.7	25.5 $\pm$ 1.27	4.8 $\pm$ 1.68
Number of young reared	102.8	9.2 $\pm$ 0.70	96.3	14.7 $\pm$ 1.11	5.5 $\pm$ 1.31
Weights of young of both sexes at the age of 28 days	9.8	37.2 $\pm$ 0.09	9.4	40.1 $\pm$ 0.08	2.9 $\pm$ 0.12

high calcium intake. Within the zone with which these experiments are concerned, the more liberal calcium intake clearly induced a more favorable nutritional response.

*Indications of adult vitality.* The appearance and behavior of our animals indicate that, as between the two diets here compared, the more liberal calcium intake resulted in a higher average of adult vitality and its maintenance over a longer time. The chief objective numerical data yielded by our type of experiment are those of the breeding records of the females. These are summarized in table 2. These findings show that

the females on the more liberal calcium intake (diet 162) matured slightly earlier, showed a longer period of ability to reproduce, bore more young and reared a higher percentage of them, and to a slightly larger average weight at weaning. Inasmuch as none of these rats reached weights which could be regarded as excessive at any age, the slightly larger average size of the parents as well as the young may probably be interpreted as further evidence of superior nutritional well-being upon the higher of the two levels of calcium intake with which these experiments deal. Again, it may be emphasized that both these are moderate levels of intake, the lower is high enough to be within the range of adequacy as ordinarily understood, and the higher is by no means radically high.

TABLE 3  
*Lengths of life on diets of different calcium content*

	DIET 16			DIET 162		
	Number of cases	C.V.	Mean with its probable error	Number of cases	C.V.	Mean with its probable error
			<i>days</i>			<i>days</i>
Males	54	24.4	651±14	49	19.7	705±13
Females	77	27.1	716±15	72	27.2	721±15

*Length of life.* The influence of added calcium upon length of life is less clear cut. Both males and females show longer average life cycles upon the higher of the two levels of calcium intake here studied. In the cases of the males the difference is very distinct, covering a large part of the difference previously found between diets A and B. In the case of the females the difference is small, and would not be regarded as significant if it stood alone. Viewed in the light of all the other data, including the fact that the females on diet 162 had reared significantly more young than those on diet 16, it is probable that for females as well as for males the more liberal calcium intake served to increase the length of life. The average lengths of life are shown in table 3. If in future it should prove possible, as we hope, to add materially to the number of these experiments upon length of life, the increased volume

of data will doubtless reduce the probable error of the means and thus permit a more conclusive evaluation of the differences.

*Regularity of nutritional response.* Together tables 1, 2 and 3 include coefficients of variability for the data of nutritional response or well-being, as shown on the two diets when compared in eleven different ways. In nine of the eleven comparisons the data for the animals on diet 162 show lower coefficient of variation (C.V.), i.e., the more liberal intake of calcium induced greater regularity of results as well as average outcomes indicative of better nutritional status.

#### DISCUSSION

In previous work with rats of the same strain, fed upon the same or similar diets (Sherman and MacLeod, '25; Sherman and Booher, '31), it has been shown that the present diet A or diet 16, while supporting growth and development at a rate within the normal range, does not induce as rapid a retention of calcium in the growing organism as does a calcium intake such as that of our diet 162; and on the level of intake afforded by diet 16 (about 0.2 per cent of calcium in the dry solids of the food mixture) the body requires a relatively long time to attain the same calcium content as that attained at the same age when the calcium intake is higher. The status supported by diet 16 is, however, well within the range of normal as this term is regularly used. It is only on much lower levels of intake, with only about half as much calcium in percentage of the food solids, that we have found evidence of subnormality attributable to low calcium content in the adult rat (Campbell, Bessey and Sherman, '35). It is also of interest to note that the calcium content of diet 16, whether calculated in percentage of the food solids or in proportion to the food calories, is higher in diet 16 than in average American family food supplies. There seems to be no room for doubt that, while the average American intake of calcium is adequate (in the accepted meaning of this word), it is below that from which optimal nutritional well-being is to be expected.

## SUMMARY AND CONCLUSION

As part of an investigation to explain the improvement of an already adequate diet, the effect of such addition of calcium carbonate as to increase the calcium content of the dry food mixture from 0.2 per cent to 0.35 per cent has been studied with rats throughout the natural life cycle and through successive generations.

This enrichment of calcium intake was followed by a more efficient utilization of the food (whether calculated on the basis of its energy value or protein content); better growth; earlier maturity; several indications of higher adult vitality; a longer period between the attainment of maturity and the onset of senility; and, in less degree, an increase in the average length of adult life, or life expectation of the adult.

Inasmuch as the infant mortality was decreased by the increase in calcium intake, the life expectation at birth was increased in greater degree than the life expectation of the adult.

That the increase of the adult life cycle was not so large with the females as with the males may be due to the fact that the females on the higher calcium intake had borne and suckled more young.

The increase in the calcium content of the already adequate diet clearly improved its nutritive value as judged by the combined criteria of growth, adult vitality, lowered death rates, and increased length of life.

We conclude that the previously reported improvement of an already adequate food supply is in part (though not solely) attributable to the enrichment of the calcium intake, and that the level of calcium intake most conducive to optimal well-being is significantly higher than that required for normal growth and maintenance.

The cooperation of the Carnegie Corporation of New York and the Carnegie Institution of Washington is gratefully acknowledged.

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## CALCIUM AND PHOSPHORUS NEEDS OF PRESCHOOL CHILDREN

AMY L. DANIELS, MARY K. HUTTON, ELIZABETH M. KNOTT,  
OLIVE E. WRIGHT AND MARY FORMAN<sup>1</sup>

*Iowa Child Welfare Research Station, State University of Iowa, Iowa City*

FOUR FIGURES

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In a recent study of the calcium needs of children of pre-school age, it was shown that under the conditions of the investigation the average calcium retentions were the same whether the children were receiving 1 pint or 1 quart of milk (Daniels et al., '34). These findings were so out of harmony with the generally accepted hypothesis that 1 quart of milk is necessary to supply the calcium for children of the ages considered (Sherman and Hawley, '22) that the work has been continued with certain slight modifications in technics and data alignment.

In calcium balance studies with children it is obvious that the relation of calcium furnished by a given quantity of milk to the total calcium of the diet differs with individuals, since in any group of children, even within a comparatively narrow age range, there will be more or less variation in size and therefore in the amount of foods other than milk taken. The calcium furnished by these foods, more particularly the fruits and vegetables, according to the findings of McClugage and Mendel ('18) and Sherman and Hawley ('22) is less available than that of milk although Rose ('20), Blatherwick and Long ('22), and Mallon, Johnson and Darby ('33) working with

<sup>1</sup> We are indebted to Miss Gladys Everson and Miss Florence Scoular for calculation of the diets and preparation of the meals.

adults, found the calcium of the vegetables tested equally well utilized. With few exceptions, the diets used for the children studied consisted of the same varieties of vegetables (carrots, tomatoes and potatoes) and fruits (prunes, apples, orange juice and banana) in very much the same proportions, therefore the availability of the calcium from these sources should be the same at a given calcium ingestion level. Variations in retentions under these conditions would seem to be due to the fact that some children needed more, either because of previous depletion or differences in potentialities of growth, or because other factors in the diet were influencing retentions, for example, an excess of calcium in relation to phosphorus or vice versa (Sherman and Pappenheimer, '21; McCollum, Simmonds, Parsons, Shipley and Park, '21) or insufficient vitamin D (Daniels, Stearns and Hutton, '29). In the study, too little vitamin D would seem to be ruled out since each child received twice daily, between breakfast and dinner and between dinner and supper, 3.5 cc. of a standard cod liver oil to which were added 4 drops of Viosterol. Furthermore, each child received daily a 10-minute exposure to the rays of a General Electric sun lamp. Both phosphorus and nitrogen ingestions and retentions have been included to determine to what extent these may have been influencing factors.

In order to meet the possible criticism of our first report that the preliminary periods were too short for metabolic adjustment, or that incomplete fecal eliminations or unavoidable inaccuracies in stool divisions may have introduced significant errors in the shorter metabolism periods, in the subsequent study the pre-periods were increased to 7 days, and two consecutive periods of 4 or 5 days each called 'double periods' were substituted for the single 5-day metabolism periods.

The food components of the diets were the same as those of the former study. In a few instances certain modifications in the amounts of given foods or in the choice of cereal foods were made for the purposes of another investigation. These apparently were without influence on the calcium and phosphorus retentions and therefore the data of these have been

included. In the interest of greater accuracy, the methods used in the preparation of the food were somewhat modified. These modifications are given in detail in the study of the protein needs of children (Daniels et al., '35). The food intakes were so adjusted that one-third of the fat, protein and carbohydrate was given at each of the three meals. In so far as possible, the milk ingestion was similarly apportioned.

The children, who were between 36 and 66 months of age were cared for as meticulously as those of the previous report. All were in good health during the period of the study, although all were not equally robust.

#### METHODS OF ANALYSIS

The analytical methods of the present study were the same as those used in the previous report (Daniels et al., '34) with the exception of a slight modification in the calcium determinations by which the calcium oxalate was precipitated at a slightly lower hydrogen-ion concentration according to the method of Washburn and Shear ('32). Tests of accuracy of the calcium and phosphorus methods by additions of known amounts to stool and food samples gave recoveries of 100.1 and 99.7 per cent, respectively. With the exception of duplicates of the urinary phosphorus, all determinations were made in triplicate.

Since retentions in the studies involving the somewhat more refined technics were found to be similar to those of the previous report, for purposes of statistical analysis the results of all calcium balance studies have been grouped according to calcium ingestions per kilogram of body weight at intervals of five between 35 mg. and 100 mg. with the corresponding phosphorus and nitrogen ingestions. The scatter diagrams showing the relation of calcium ingestions to retentions indicate the double periods as well as the first and second consecutive periods of the former study.

The higher calcium retentions of childhood are concerned chiefly with skeletal growth; thus it seemed possible that retentions in relation to ingestions based on height might give

more accurate information regarding children's needs than retentions based on weight. In the study, therefore, the retentions have been grouped also in relation to height between ingestions of 7.0 mg. and 14.9 mg. per centimeter at intervals of ten.

#### RESULTS

A consideration of the average calcium retentions at the various levels of ingestion tested (table 1) gives little information concerning the needs of children of the ages studied. With the exception of two cases at the upper level of ingestion, the highest average retention, 12 mg. per kilogram, was obtained with ingestions between 60 and 64 mg. per kilogram, whereas with ingestions between 90 mg. and 94 mg. per kilogram the average retention was somewhat less, 9 mg. per kilogram. It will be noted that the range of retentions is fairly comparable at each ingestion level above 40 to 44 mg.

Optimum retentions at a given ingestion level, which would seem to be indicative of the availability of the calcium, were not directly related to the ingestions, within the limits of the study. For example, retentions of 15 mg. per kilogram were obtained with ingestions of 45 mg., 65 mg., 85 mg., and 95 mg. per kilogram, respectively, while retentions of 14 mg. were found with ingestions of 70 mg. as well as 80 mg. per kilogram. At the ingestion level of 35 to 39 mg., both average and optimum retentions were low, but not lower than certain retentions obtained with higher ingestions. Whether these lower retentions were due to too little calcium in the diet or to the fact that the children needed less is not clear. This lower ingestion level included five balance studies with two children who were unusually robust and considerably (from 5.6 to 19.4 per cent) above the theoretical weight for height. Estimating the retentions of these children on the basis of their theoretical weights, brings the average well within the range of the groups above.

Calcium ingestions based on height (table 2) also seem to indicate that sufficient calcium was given at all levels of ingestion tested. The highest retention, 2.8 mg. per centimeter,

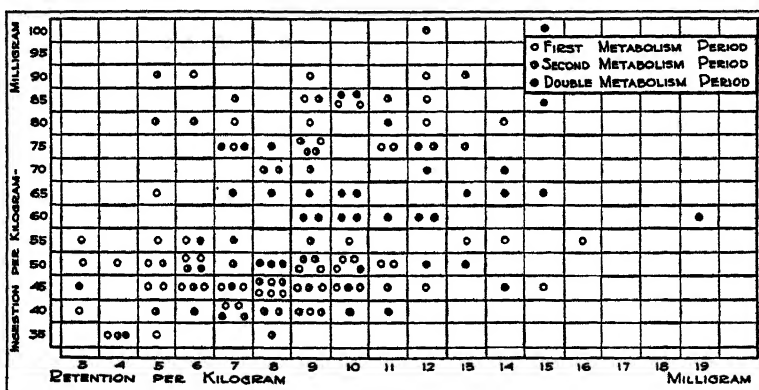
TABLE 1  
*The relation of calcium retention to calcium ingestion based on weights of the children studied*

CALCIUM INGESTION PER KILOGRAM	NUMBER OF CASES	AVERAGE AGE	AVERAGE WEIGHT	CALCIUM RETENTION			PHOSPHORUS INGESTION		NITROGEN INGESTION	
				Average per kilogram	Standard deviation	Range per kilogram	Average per kilogram	Range per kilogram	Average per kilogram	Range per kilogram
mg.		yr. mo.	kg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
95-100	2	3- 8	13.57	14	..	12-15	94	91- 96	620	580-680
90- 94	5	4- 5	15.16	9	..	5-13	95	86-101	610	540-680
85- 89	10	4- 8	15.76	10	2	8-15	88	79-100	590	490-700
80- 84	7	4-11	16.69	9	3	5-14	81	75- 90	520	460-570
75- 79	13	5- 0	17.71	10	2	7-13	80	73- 89	570	470-610
70- 74	5	4- 9	17.80	10	..	8-14	79	74- 87	540	470-660
65- 69	8	4-11	18.05	10	3	5-15	73	63- 84	510	400-610
60- 64	8	4- 6	18.51	12	3	9-19	73	64- 78	540	470-630
55- 59	10	3- 6	13.85	9	4	3-16	70	63- 76	520	440-570
50- 54	21	4- 8	15.58	8	2	4-12	69	59- 82	520	420-630
45- 49	24	5- 1	17.22	9	2	5-15	62	53- 76	460	390-550
40- 44	12	5- 1	18.50	8	2	3-11	60	51- 67	460	390-530
35- 39	5	5- 7	22.50	5	..	4- 8	54	52- 59	420	390-430

TABLE 2  
*The relation of calcium retention to calcium ingestion based on heights of the children studied*

CALCIUM INGESTION PER CENTIMETER	NUMBER OF CASES	AVERAGE AGE	AVERAGE HEIGHT	CALCIUM RETENTION			PHOSPHORUS INGESTION		NITROGEN INGESTION	
				Average per centimeter	Standard deviation	Range per centimeter	Average per centimeter	Range per centimeter	Average per centimeter	Range per centimeter
mg.		yr. mo.	cm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
14.0-14.9	6	3- 9	93.6	1.8	0.36	1.2-2.2	13.6	13.0-14.0	90.5	81.5- 96.5
13.0-13.9	28	4-10	102.9	1.5	0.43	0.7-2.6	13.8	11.1-15.5	92.4	75.8-109.2
12.0-12.9	11	4-11	106.6	1.7	0.41	1.0-2.3	13.2	11.8-14.5	88.4	72.8-104.7
11.0-11.9	2	4- 8	104.7	1.8	....	1.8-1.9	13.5	13.4-13.6	100.3	98.6-101.9
10.0-10.9	8	4-10	104.6	2.0	0.39	1.5-2.7	12.1	9.9-13.4	87.8	64.9-109.2
9.0- 9.9	3	4- 2	100.1	2.1	....	1.7-2.8	12.0	11.4-12.8	82.7	74.8- 92.2
8.0- 8.9	30	4- 4	98.5	1.3	0.45	0.4-2.3	10.9	9.3-12.4	81.1	64.5- 98.5
7.0- 7.9	42	5- 1	103.9	1.3	0.40	0.6-2.4	10.3	9.1-12.4	77.1	62.8- 96.0

was obtained with an ingestion of 9.9 mg. per centimeter. At ingestions between 14.0 and 14.9 mg., and 11.0 to 11.9 mg., the same average retentions were obtained, namely, 1.8 mg. per centimeter, which was slightly lower than the average retentions of 2.0 and 2.1 obtained with ingestions between 10.0 and 10.9 mg. and 9.0 to 9.9 mg. per centimeter, respectively. The upper range of retentions at these ingestion levels was also quite similar, namely, 2.7 and 2.8 mg., respectively. With an ingestion of 14.0 to 14.9 mg., the highest of the series one child retained only 2.2 mg., slightly less than the optimum,



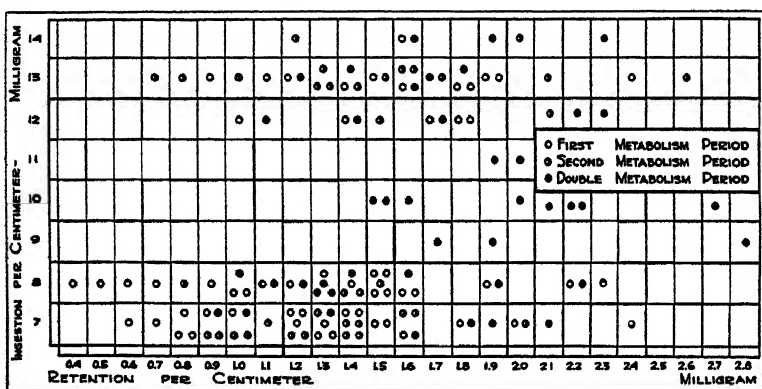
SCATTER DIAGRAM No. I  
THE RELATION OF CALCIUM RETENTION  
TO CALCIUM INGESTION  
BASED ON WEIGHTS OF THE CHILDREN STUDIED

2.4 mg. per centimeter, obtained at the lowest ingestion, 7.0 to 7.9 mg. per centimeter.

The scatter diagrams depicting the calcium retentions estimated on both weight and height (scatter diagrams 1 and 2) even more clearly than the tables, indicate that under the conditions of the study the calcium needs of children can be met by lower ingestions than we have hitherto considered necessary. Seventy-four per cent of all the children studied retained 10 mg. per kilogram or less. Of these 63 per cent received 1 pint of milk, and 37 per cent between a pint and a quart. Based on height, 69 per cent of the children studied retained



1.6 mg. per centimeter or less, irrespective of the amount ingested, 32 per cent of these being among those receiving 10 mg. or more per centimeter from diets containing more than a pint of milk, and 68 per cent among those receiving less than 10 mg. per centimeter. High retentions estimated either on weight or height are found at all ingestion levels. Among those receiving either a pint or a quart of milk are a number of children with high retentions, who were studied during several metabolism periods, although these were not consecutive. Estimating these high retentions, either on the



SCATTER DIAGRAM No. II  
THE RELATION OF CALCIUM RETENTION  
TO CALCIUM INGESTION  
BASED ON HEIGHTS OF THE CHILDREN STUDIED

basis of weight or height, of the individual children studied, brings the percentage of retentions above 10 mg. per kilogram, and 1.6 mg. per centimeter to 16 per cent and 18 per cent, respectively.

In a previous report regarding the protein needs of children (Daniels et al., '35) it was suggested that the wide variations in the nitrogen retentions at given ingestions above the minimum were due either to previous depletion of the children studied or to differences in potentialities of growth. The data of the present investigation suggest that calcium retentions also are related to the physical condition of the children studied as well as to the growth pattern. On the other hand,

in the diets of the children, there were more or less differences in the amounts of phosphorus and nitrogen ingested. Studies with animals have shown that any considerable disparity between the calcium and phosphorus ingestions, resulting in an excess of either, may materially influence the calcium retentions (Hart, Steenbock and Elvehjem, '24; Shohl et al., '33). The addition of vitamin D or exposure to the ultra-violet rays of the sun or of a mercury quartz lamp tend to nullify the results (Hart, Steenbock and Hoppert, '21; Templin and Steenbock, '33). Similarly, the untoward effects of a diet potentially acid or basic is modified by the inclusion of sufficient amounts of vitamin D (Morgan, '34). From experiments with animals, therefore, one may conclude tentatively that any imbalance of these two elements in the diets of the children studied would not have affected materially the results, provided, of course, enough of each were supplied, since the vitamin D given was controlled, and in so far as is known, adequate. That such was the case is shown by the fact that similar retentions were obtained at quite different ingestion levels of the three metabolites, calcium, phosphorus and nitrogen, which are conceded to be interrelated. Space allows for only a few examples at given ingestion levels (table 3).

Comparing the retentions resulting from dissimilar ingestion levels, it is obvious that the amounts and relationships of these metabolites were not the determining factors in the amount of calcium retained, since comparable retentions were obtained with very different ingestions. Nor can the availability of the calcium of the diet indicated by the percentage of calcium from milk be the determining factor.

It would seem that the needs of the children differed at the particular time of the study. Growth in childhood is a continuous, but not necessarily a symmetrical process. During a given period, some tissues may be growing at a more rapid rate than others and requiring different amounts of the substance tested, or previous to the study certain of the children may have received adequate amounts of some substances and less than the optimum amounts of others; thus the

TABLE 3

*Similar retentions of calcium, phosphorus and nitrogen at different levels of ingestion*

NAME	DATE	AGE	WEIGHT	PER CENT OVER + OR UNDER — THEORETICAL WEIGHT <sup>1</sup>	RETENTION PER KILOGRAM			RETENTION RATIO $\left(\frac{P-99\%Ca}{N}\right) \times 1.94$	INGESTION PER KILOGRAM			TOTAL CALCIUM FROM MILK
					Calcium	Phosphorus	Nitrogen		Calcium	Phosphorus	Nitrogen	
		yr. mo.	kg.		mg.	mg.	mg.		mg.	mg.	mg.	per cent
B. S.	1/30/34	4-5	15.41	— 8.0	14.3	10.8	70	20:1	72	83	570	85
L. B.	2/1/34	5-2	16.59	— 3.7	14.2	11.1	76	19:1	69	79	580	85
L. B.	1/10/34	5-1	16.39	— 2.1	13.5	10.8	96	25:1	47	55	410	80
F. W.	6/12/32	3-1	14.04	+ 2.1	13.0	10.5	106	27:1	92	89	540	91
C. H.	11/14/34	4-9	15.78	— 7.9	12.4	9.7	107	31:1	54	77	630	72
B. S.	2/21/34	4-6	15.95	— 4.9	12.0	9.7	99	27:1	73	87	660	82
P. D.	5/2/34	4-10	17.09	+ 6.1	11.7	7.8	80	42:1	75	84	810	90
D. B.	11/14/32	4-3	16.10	+ 0	11.3	7.6	79	44:1	51	65	470	73
D. B.	12/2/32	4-4	17.00	+ 4.7	11.0	8.3	72	27:1	46	63	430	77
C. P.	6/11/33	6-1	17.50	— 4.8	11.8	8.3	67	29:1	81	75	460	90
J. E.	10/1/34	3-11	15.78	+ 8.1	10.0	7.7	92	35:1	86	83	570	90
D. G.	11/14/34	4-8	19.33	+ 7.4	10.5	7.7	89	39:1	43	63	550	73
G. O.	5/12/33	6-1	16.65	— 8.4	9.6	9.1	115	27:1	87	100	690	84
C. P.	6/23/33	6-2	17.50	— 4.8	9.2	9.1	114	25:1	80	86	570	89
N. L.	6/10/34	3-2	14.52	— 0	8.5	6.2	80	42:1	54	76	570	77
L. T.	3/5/33	4-10	14.96	— 12.0	8.7	6.2	76	42:1	54	73	590	75
A. C.	2/3/33	5-5	18.00	+ 12.5	7.3	11.2	103	14:1	44	62	480	73
A. C.	3/19/33	5-6	18.00	+ 9.1	7.4	11.0	112	15:1	44	60	450	77
R. H.	3/5/33	5-0	18.30	+ 5.8	6.9	5.6	56	27:1	44	60	480	75
B. S.	10/4/33	4-2	12.90	— 5.1	6.5	5.6	57	25:1	57	63	440	81

<sup>1</sup>Theoretical weight estimated from: 'Physical traits of young children.' Iowa Child Welfare Research Station, '29. Am. J. Dis. Child., vol. 38, p. 541.

relationship between calcium and phosphorus on the one hand, and phosphorus and nitrogen on the other would not be constant. When skeleton, muscle and glandular tissue are growing at the optimum rate as in infancy, during adolescence, or convalescence from a wasting disease, a fairly constant ratio between calcium, phosphorus and nitrogen would be expected. During periods of less rapid growth, somewhat different ratios would obtain. Since phosphorus is necessary for the development of bone as well as muscle and glandular tissue, it seemed that the phosphorus retention might be indicative of the nutritional status of the child, especially when it is considered in relation to the calcium and nitrogen retentions. The data of the study, therefore, have been grouped in relation to the phosphorus retentions at intervals of ten between 2.5 mg. and 14.4 mg. per kilogram, with the corresponding nitrogen and calcium retentions (table 4). The relation of phosphorus to nitrogen after the calcium needs for bone growth have been satisfied, called the retention ratio  $\left( \frac{N}{P - \frac{99\% Ca}{1.94}} \right)$  also has been determined for each retention level.

At a given phosphorus retention level, these retention ratios were found to be surprisingly though not absolutely constant, averaging 17:1 at the highest retentions of 13.5 to 14.4 mg. per kilogram, and gradually increasing as the phosphorus retention decreased until at retentions of 3.5 to 4.4 mg. per kilogram the ratio was 34:1. At 2.5 to 3.4 mg. per kilogram, slightly less phosphorus than enough to combine with the calcium for bone formation was retained. These low phosphorus retentions were obtained with two unusually robust children who were considerably (29 per cent) above average weight.

The phosphorus ingestions, apparently, were not a factor in influencing these low retentions since high retentions were observed quite as frequently among children receiving low ingestions. This is shown by the scatter diagrams depicting the relation of phosphorus retentions to phosphorus ingestions. In general, low phosphorus retentions were coexistent

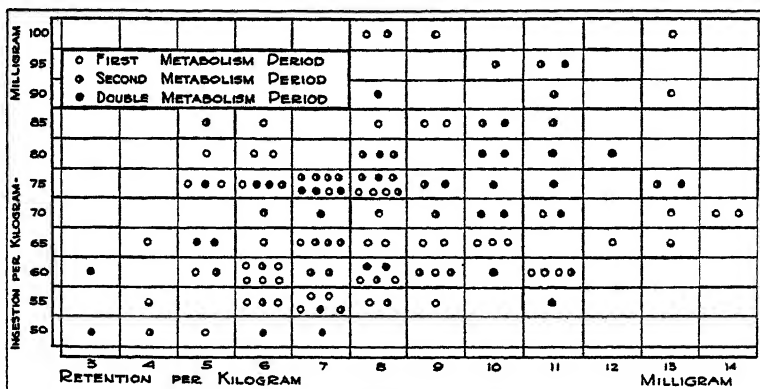
TABLE 4  
*The interrelation of phosphorus, calcium and nitrogen retentions based on comparable phosphorus retentions*

PHOSPHORUS RETENTION RANGE PER KILOGRAM	NUMBER OF CASES	AVERAGE AGE	AVERAGE WEIGHT	PER CENT OVER + OR UNDER - THEORETICAL WEIGHT <sup>1</sup>	AVERAGE RETENTION PER KILOGRAM			RETENTION RATIO $\left(\frac{P-99\%Ca}{N}\right)$ 1.94	AVERAGE INGESTION PER KILOGRAM		
					Phosphorus	Calcium	Nitrogen		Phosphorus	Calcium	Nitrogen
mg.		yr. mo.	kg.		mg.	mg.	mg.		mg.	mg.	mg.
13.5-14.4	2	3-7	15.29	+ 5.5	14.0	11.8	135	17:1	71	54	540
12.5-13.4	6	4-0	14.73	- 2.4	13.2	13.0	108	16:1	80	67	530
11.5-12.4	2	4-0	14.53	- 6.5	12.1	11.9	108	18:1	76	63	560
10.5-11.4	13	4-8	15.81	- 0.7	11.0	11.6	97	19:1	76	66	530
9.5-10.4	12	4-6	16.06	- 2.4	9.9	10.6	101	22:1	77	66	560
8.5-9.4	12	4-10	17.13	+ 1.4	8.9	8.5	105	23:1	73	62	510
7.5-8.4	24	4-11	17.12	- 1.0	7.9	8.8	92	27:1	73	63	520
6.5-7.4	21	4-11	17.25	+ 2.2	6.9	8.3	77	29:1	67	51	490
5.5-6.4	19	4-8	16.86	+ 2.3	5.9	7.6	70	34:1	68	58	500
4.5-5.4	10	4-5	17.44	+ 4.9	5.1	7.3	64	46:1	72	67	500
3.5-4.4	3	5-4	20.70	+ 5.3	4.0	4.3	59	34:1	59	41	440
2.5-3.4	2	5-0	21.62	+ 29.2	3.0	6.6	47	....	58	50	430

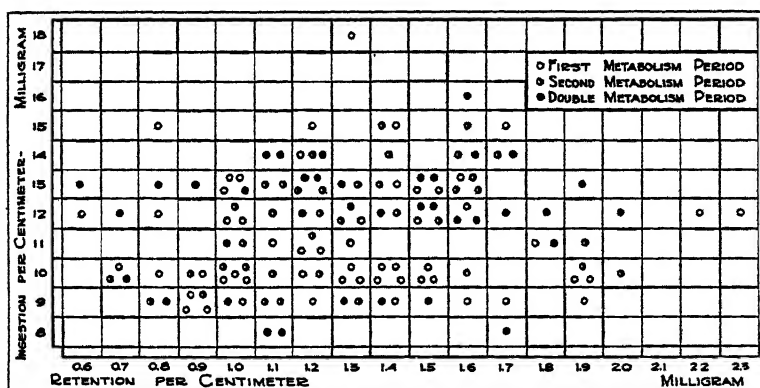
<sup>1</sup> Theoretical weight estimated from: 'Physical traits of young children.' Iowa Child Welfare Research Station, '29.  
Am. J. Dis. Child, vol. 38, p. 541.

with low nitrogen retentions, indicating that the soft tissues of these children were well developed.

Calcium retentions, on the other hand, with the exception of those at the higher levels, remained more nearly constant,



SCATTER DIAGRAM No. III  
THE RELATION OF PHOSPHORUS RETENTION  
TO PHOSPHORUS INGESTION  
BASED ON WEIGHTS OF THE CHILDREN STUDIED



SCATTER DIAGRAM No. IV  
THE RELATION OF PHOSPHORUS RETENTION  
TO PHOSPHORUS INGESTION  
BASED ON HEIGHTS OF THE CHILDREN STUDIED

between 6.6 and 8.8 mg. per kilogram, suggesting that the high retentions either of phosphorus or calcium are the result of previous depletion. Further evidence of this is suggested by the retention ratios obtained by Jeans and Stearns ('33)

during infancy, by Stearns and Moore ('31) in under-nutrition and subsequent recovery in a 3-year-old child, and by Luthje and Berger ('04) during convalescence from typhus in an adult. Low phosphorus retentions, when coexistent with high retention ratios apparently are indicative of more nearly physically fit children.

#### COMMENT

In a previous study it was postulated that 1 pint of milk when included in a diet otherwise adequate would supply sufficient calcium for the child of preschool age. The findings in the present study in which the calcium needs of children have been considered in relation to ingestion based on both weight and height, verify our former results and indicate that from 45 to 50 mg. of calcium per kilogram, or 7 to 8 mg. per centimeter, are sufficient for the age group studied, provided the calcium is available and sufficient vitamin D is allowed.

The phosphorus needs of children of the age group studied seemingly can be met by 55 to 60 mg. per kilogram, or between 9 and 10 mg. per centimeter, depending on the presence of an adequate amount of vitamin D. Calcium retentions between 7 and 9 mg., and phosphorus retentions between 6 and 9 mg. per kilogram would seem to be normal for well-developed children of the age studied.

#### SUMMARY

Calcium, phosphorus and nitrogen balance studies with children of preschool age receiving diets composed of the same varieties of food but differing in amounts and therefore in the quantities of the inorganic substances contained therein have been considered from the standpoint of both height and weight. The vitamin D in all cases was controlled, and in so far as is known, adequate, thus ruling out any variation which might accrue from too little.

Wide variations in both calcium and phosphorus retentions were obtained at similar ingestion levels. High retentions were interpreted as being due to previous depletion.

The retention ratios of the nitrogen to phosphorus after the phosphorus needs for bone growth had been satisfied ( $\frac{N}{P - \frac{99\% Ca}{1.94}}$ ) grouped according to the phosphorus retentions were found to increase as the phosphorus retentions decreased and were roughly proportional to the nutritional status of the child as indicated by the variation from the theoretical weight for height of the group considered. Within the limits of the study, the retentions were uninfluenced by the ingestions. The majority of the calcium retentions were found to be between 6 and 10 mg. per kilogram, irrespective of the ingestions.

The results of the study indicate that the calcium needs of normal children of preschool age can be met by foods containing between 45 and 50 mg. of calcium per kilogram, or between 7 and 9 mg. per centimeter, provided sufficient vitamin D is allowed. Phosphorus needs, on the other hand, can be met by food containing between 60 and 70 mg. per kilogram, or between 9 and 11 mg. per centimeter.

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# THE EFFECT OF DEFICIENT DIETS ON THE TOTAL ASH, CALCIUM AND PHOSPHORUS CONTENT OF BONES<sup>1</sup>

RUTH YEAGER AND JET C. WINTERS

*Department of Home Economics, University of Texas, Austin*

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Winters, Smith and Mendel ('27) published the results of an investigation dealing with the effects of dietary deficiencies on the growth of certain body systems and organs. This study dealt with calorie, protein and mineral deficiencies which were so drastic that growth was practically inhibited; young animals gained only 5 to 10 gm. during a 40-day period. Among others, the observation was made that persistent skeletal growth occurred on all the diets used and was manifested by an increase in body and tail length and an increase in the length of the leg bones and skull. The amount of this skeletal growth, however, varied with the different types of deficiency. The present report describes the total ash, calcium and phosphorus in the bones of animals stunted in the various ways; comparison is made in these respects with normal rats of the same age and with normal rats of the same weight. An attempt is made to relate the mineral content of the bones of the animals stunted in different ways to linear measurement and to weight; and to discover what, if any, differences in the total ash, calcium, and phosphorus content of bone result from the different kinds of dietary deficiency.

<sup>1</sup>From a thesis submitted in partial fulfillment of the requirements for the degree of master of science in home economics, to the faculty of the graduate school, University of Texas, Austin, Texas.

## EXPERIMENTAL PROCEDURE

Six groups of rats were used; with one exception each group consisted of twenty-five male animals. There were two control groups, one of which consisted of animals of approximately the same age as the stunted groups (referred to as age controls) and the other of animals of approximately the same weight (referred to as weight controls). The age controls, of which there were only five, were, of course, much heavier and the weight controls much younger than the stunted animals. The figures obtained from the weight control groups were used as a base for estimating percentage increases in weight, length and mineral content of the bones during the stunting period, i.e., it was assumed that the findings for the weight control group were representative of what would have been true for the stunted groups had it been possible to weigh, measure and analyze their bones at the beginning of the stunting period. Allowance for such small increases as might normally occur with a weight increase of 5 gm. (the average increase of the stunted groups) was made by using a weight-control group 5 gm. heavier, on the average, than the stunted group were at the beginning of the experiment. For the stunted groups, animals were started on the experiment at ages varying from 21 to 25 days, but always at a weight of 40 gm. During a 40-day period of stunting no animal gained more than 10 gm., and no group showed an average weight increase of more than 5 gm. The four types of stunting used were 1) low calorie, 2) low protein, 3) low lysine, and 4) low mineral. For the diets and the experimental procedure used to bring about restriction of growth the reader is referred to the report of Winters, Smith and Mendel ('27).

Since all the breeding rats used for the production of the litters involved in the experiment were on the same stock diet, and the rats, after separation from the mother, were kept on the same diet until ready for the experiment, there was little variation in the opportunity for storage of minerals. In distributing litter mates among the experimental and con-

trol groups, care was taken to get as equal a distribution as possible with the large number of animals involved.

The right fore and hind legs of the animals were used for the weight, linear measurement and mineral analysis figures here reported. These bones were prepared by carefully cutting through the joints at the shoulder and thigh and cutting away the outside skin. This preparation was placed in a 2 per cent soap powder solution and kept at a temperature of 95°C., for a period of 1 hour. The skin is then easily pulled away from the bones. The bones were dried at room temperature for a period of 30 days before being weighed and measured.

In order to get samples for analysis, the leg bones of the rats of each group were pooled, folded in cloths, crushed between the jaws of large pliers and then pounded to insure proper breaking. Each of the six composites thus obtained were extracted with hot 95 per cent alcohol for a period of 18 hours and with ether for an equal period of time. They were then freed from ether and dried in an electric oven at 96°C. for 1 hour. Weighed samples of the alcohol-ether extracted bones from each group of rats were ashed by first charring over a low flame and then heating in a gas muffle furnace until constant weight was obtained. This ash was then analyzed for calcium and phosphorus. All determinations were done in triplicate.

The modified method of McCrudden was used to determine the calcium. To determine the phosphorus, the method from the Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists ('30) was used.

#### RESULTS OF EXPERIMENTAL WORK

Table 1 gives the percentage of total ash and of calcium and phosphorus in the alcohol-ether extracted bones of rats held at constant body weight by means of various dietary adjustments, together with similar data for normal rats, 1) of the same age, and 2) of the same weight.

TABLE 1

*Percentage of total ash, calcium and phosphorus in bones of animals stunted by dietary deficiency*

DIETS	TOTAL ASH	CALCIUM	PHOSPHORUS
Age controls, same age as stunted animals	61.02	23.24	10.95
Weight controls, same weight as stunted animals	51.12	18.20	9.28
Low calorie	58.72	22.02	10.06
Low protein	58.34	21.43	10.31
Loy lysine (gliadin)	57.90	21.50	9.27
Low mineral	45.33	15.65	8.58

## DISCUSSION OF RESULTS

Winters, Smith and Mendel ('27) (table 2) showed that the various leg bones of rats stunted in different ways (low calorie, low protein, incomplete protein, and low mineral diets) increased 10 to 23 per cent in length during a period of 40 days. There was little variation with the type of diet used,

TABLE 2

*Percentage increase in length of leg bones of rats stunted by deficient diets<sup>1</sup>*

	LOW CALORIE	LOW PROTEIN	GLIADIN	LOW SALT
Humerus	13	10	13	10
Ulna	14	13	15	12
Radius	17	18	18	17
Femur	23	18	18	18
Tibia	15	13	15	13

<sup>1</sup> The percentage increases are calculated by comparing the average length of the bones of the animals in the stunted groups with the average length of the bones of normal animals of the same weight.

the leg bones of the low calorie animals tending to be slightly longer and those of the low mineral animals slightly shorter than the others.

The same investigators found (table 3) that the increases in the weight of the leg bones were, except for the low salt rats, much more pronounced than increases in length and that there was considerable variation with the different kinds of stunting.

Inasmuch as increase in weight of bones is due largely to increase in mineral constituents, the large differences between changes in weight and length characteristic of all the deficiencies except that of minerals may be taken to mean that the percentage of ash had been augmented in the bones during the stunting period. An increase in percentage of minerals is characteristic of normally developing bones. The

TABLE 3

*Percentage increase in weight of leg bones of rats stunted by deficient diets<sup>1</sup>*

	LOW CALORIE	LOW PROTEIN	GLIADIN	LOW SALT
Humerus	74	54	54	5
Ulna and radius	61	51	42	0
Femur	90	65	71	20
Tibia and fibula	63	50	61	14

<sup>1</sup> The percentage increases are calculated by comparing the average weight of the bones of the animals in the stunted group with the average weight of the bones of normal animals of the same weight.

TABLE 4

*Percentage increase in total ash, calcium and phosphorus of leg bones of rats stunted by deficient diets<sup>1</sup>*

KIND OF STUNTING	TOTAL ASH	CALCIUM	PHOSPHORUS
Low calorie	7.60	3.81	0.78
Low protein	7.22	3.22	1.03
Low lysine (gliadin)	6.78	3.29	0.01
Low salt	— 5.79	— 2.56	— 0.70

<sup>1</sup> The percentage increases are calculated by comparing the percentages of total ash, calcium and phosphorus in the bones of the stunted groups with similar percentages in the bones of normal animals of the same weight.

percentage increases in total ash, calcium and phosphorus are given in table 4 and bear out the idea of increase in the mineral content of bone during the stunting period.

Since the bones of the low mineral animals show such a slight increase in weight in comparison with those of the animals stunted in other ways, and at the same time show an almost equal increase in length, it may be concluded that mineral deficiency does not bring about any greater skeletal

stunting than other deficiencies, but that it does result in bones that are poorer in mineral content. Table 4 shows the decrease in total ash, calcium and phosphorus content of the bones of the animals on the mineral-deficient diet; almost half the decrease is due to loss of calcium. These results may be taken as supplementing those of Sherman and Booher ('31) who found no difference in the growth or physical appearance of rats kept, for varying lengths of time, on diets in which calcium was the sole significant variable. The calcium content of the bodies of such rats, however, varied directly with the calcium intake.

Comparison may also be made of the mineral content of the bones of the stunted animals with that of animals of the same age. From table 1 it can be calculated that the leg bones of rats that were kept on a purified, but supposedly adequate, diet during a period of time equivalent to the stunting period showed an increase of 10 per cent in total ash, 5 per cent in calcium, and 1.68 per cent in phosphorus. In no case do the bones of stunted animals show as great an increase in percentage of total ash, calcium, or phosphorus as do those of these normal animals of the same age. It is evident that the leg bones of animals stunted by means of low calorie, low protein, or incomplete protein diets have a much higher percentage of total ash, calcium and phosphorus than those of the younger animals of the same weight, but a lower percentage than those of the heavier animals of the same age. Stunting by means of a low mineral diet, however, results in loss of calcium, phosphorus, and total ash so that the leg bones of such animals have a much lower percentage of total ash, calcium and phosphorus than those of animals stunted in other ways. It is evident that animals on certain types of deficient diets store calcium and phosphorus even when their general growth is considerably below normal but that the storage is at a subnormal rate. Sherman and McLeod ('25) found that the bodies of rats kept on a diet adequate except for the fat-soluble vitamins contained, at 90 days, a percentage of calcium which was slightly more than was characteristic of normal rats of the same weight but less than half as

great as that characteristic of normal rats of the same age. Similar results were found on a diet deficient in vitamin B, on one deficient in cystine and on one drastically deficient in calories. The same investigators report that animals on a low calcium diet actually lose body calcium.

In bones all of the phosphorus and most of the calcium is stored as calcium phosphate. There are, however, small amounts of other calcium salts, particularly calcium carbonate. The question arises as to whether calcium and phosphorus are always stored in bones in definite ratio or whether the ratio characteristic for animals on a normal diet may be upset by deficient diet. Sherman and Quinn ('26) pointed out that in the bodies of rats on an adequate diet, calcium increased at a more rapid rate than phosphorus. That this holds true for the bones described above is indicated by the finding that the Ca:P ratio in bones of 25-day-old normal rats, on a purified, but supposedly adequate diet, is 1.96, whereas that for rats kept on the same diet until approximately 65 days of age is 2.12. The ratio is increased to 2.30 for rats 125 days of age, according to figures by Brooke, Smith and Smith ('34). The Ca:P ratio in the bones of the animals stunted by low calories and low protein is similar to that of rats of the same age rather than the same weight, being 2.18 and 2.08, respectively. The ratio is somewhat higher for the gliadin stunted animals, i.e., 2.31. This may be accounted for by the lesser supply of phosphorus brought about by substituting a simple protein (gliadin) for a phospho-protein (casein). The Ca:P ratio for the low salt animals is 1.82, showing a larger storage of phosphorus in proportion to calcium than is characteristic even of normal rats of the same weight. Brooke, Smith and Smith ('34) determined the calcium and phosphorus content of the leg bones of two groups of 125-day-old rats; one group had been adequately fed and the other kept on a low salt ration similar to the one used in the present experiment. From their figures Ca:P ratios of 2.30 and 2.07 for the adequately fed and low salt animals, respectively, may be calculated. By determining the amount



of calcium present as carbonate, these investigators showed that the proportion of calcium present in bones as carbonate is less in animals given a low salt diet than in those adequately fed. They interpret the shift in the phosphate to carbonate ratio as being due to the fact that in rats on a low salt ration, calcium is taken from the carbonate of the bones to a greater extent than from the phosphate. The figures obtained in this experiment lend themselves to the same interpretation, but there is also the possibility that a dietetic factor is involved. In the low salt diet a certain amount of phosphorus was supplied by the casein but very little calcium was probably available. Inasmuch as no mineral analysis, except total ash, was made on the diet, no quantitative figures are available. Whether or not the dietetic factor plays any great part could be found by repeating the experiment, using a protein that does not contain phosphorus in the low salt ration. The high Ca:P ratio in the bones of the gliadin-stunted animals lends, as pointed out above, some support to the idea that differences in the amounts of calcium and phosphorus supplied by the diet may affect the Ca:P ratio in the bones.

#### SUMMARY

The right fore and hind leg bones of groups of young, male, albino rats which had been kept at stationary body weight for a period of 40 days by means of deficient diets (low calorie, low protein, incomplete protein, and low salt) were pooled and analyzed for total ash, calcium and phosphorus. Similar analyses were made on the bones of normal rats of the same weight (weight controls) and on the bones of normal rats of the same age (age controls). The percentages of total ash, calcium and phosphorus in the bones of the stunted rats are then compared with those in the bones of the controls; the results obtained by comparison with the weight controls are taken to represent the amount of the mineral stored during the stunting period.

The bones of animals stunted by means of calorie, protein and lysine deficiency showed a much larger percentage of total ash, calcium and phosphorus than was present in the bones of normal animals of the same weight but a smaller percentage in each case than is found in the bones of normal animals of the same age. Stunting by means of a low salt diet resulted in loss of total ash, calcium and phosphorus so that the percentages in the bones of animals so stunted are considerably smaller than those characteristic of normal animals of the same weight and greatly reduced in comparison with those of normal animals of the same age.

There is some indication that the Ca:P ratio in bones depends, to some extent at least, on the amounts of calcium and phosphorus present in diet.

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# THE AUGMENTATION OF THE TOXICITY OF FLUOROSIS IN THE CHICK BY FEEDING DESICCATED THYROID<sup>1</sup>

PAUL H. PHILLIPS, HONORA ENGLISH AND E. B. HART

*Department of Agricultural Chemistry, University of Wisconsin, Madison*

ONE FIGURE

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Since fluorine has been shown to inhibit normal respiration in certain tissues of the body by Phillips, Stare and Elvehjem ('34), effort was directed to preliminary experiments to see if the addition of metabolic stimulants would not counteract the influence of NaF feeding. Mild doses of desiccated thyroid and 2,4-dinitrophenol fed at the rate of 5 to 6 mg. daily with 0.07 per cent NaF were without beneficial effect in deterring the appearance and development of fluorine toxicosis during the rapid growing period of the chick (Phillips, '34). On the other hand 0.10 per cent of NaF fed in combination with toxic levels of desiccated thyroid shortened the survival period of the chicks. It appeared that if desiccated thyroid were fed in sufficient quantity to influence the basal metabolism its toxicity was appreciably enhanced by NaF, or vice versa. The present study was undertaken with the view of attempting to determine the influence of NaF poisoning upon animals given desiccated thyroid in doses sufficient to cause a reaction but which were non-toxic in short periods.

In preliminary experiments the tolerance of growing chicks to desiccated thyroid ingestion was established (Phillips, English and Hart, '35). It was found that 0.5 per cent was

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definitely toxic within 6 weeks. One-tenth, 0.2, and 0.3 per cent levels were without obvious harm except for a response in blood cholesterol. The effects of NaF upon growth in the chick were found to be a slight retardation at a level of 0.07 per cent while 0.09 and 0.10 per cent levels were sufficient to clearly retard growth within a period of 6 weeks.

#### EXPERIMENTAL

Two successive experiments were made. Day-old White Leghorn chicks were used in each case. The basal ration no. 351 (Kline, et al., '34) was composed of:

Yellow corn	58 parts
Wheat middlings	25 parts
Casein	12 parts
NaCl	1 part
CaCO <sub>3</sub>	1 part
Yeast	1 part
Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub>	1 part
Cod liver oil	1 part
Dried liver residue	10 parts

NaF and desiccated thyroid were administered singly and in combination according to the following plan.

<i>Experiment 35A</i>	
<i>Lot</i>	<i>Ration</i>
I	Basal ration only
II	Basal ration + 0.22 per cent NaF (0.1 per cent F)
III	Basal ration only with intraperitoneal injections of NaF
IV	Ration of lot II with 0.2 per cent desiccated thyroid
V	Ration of lot II with 0.4 per cent desiccated thyroid
VI	Ration of lot II with 0.6 per cent desiccated thyroid
VII	Basal ration + 0.4 per cent desiccated thyroid
VIII	Basal ration + 0.6 per cent desiccated thyroid

The results from this experiment suggested that the levels of fluorine and desiccated thyroid were sufficient to enhance their combined toxicity over that of either alone. Milder doses were then used in a second experiment with chicks.

*Experiment 35B*

<i>Lot</i>	<i>Ration</i>
I	Basal ration only
II	Basal ration + 0.2 per cent NaF (0.09 per cent F)
III	Basal ration only with intraperitoneal injections of NaF
IV	Ration of lot II with 0.05 per cent desiccated thyroid
V	Ration of lot II with 0.10 per cent desiccated thyroid
VI	Ration of lot II with 0.20 per cent desiccated thyroid
VII	Basal ration with 0.20 per cent desiccated thyroid

The original plan for intraperitoneal injections of NaF was to inject a 0.9 per cent solution of NaF at a level of one-half of the quantity of fluorine ingested by lot II. This level was acutely toxic and killed the birds within 1 to 2 hours. Replacements were made at once and a graded series of injections started. The levels used were 8, 16, 32 and 64 mg. of F per kilogram of body weight. A group of 6-week-old White Leghorn chicks were likewise injected. Physiological saline injections were made daily in several birds to serve as control animals for the lot. In experiment 35B all injections were made at the rate of 34 to 40 mg. of fluorine per kilogram of body weight.

Feed consumption records, growth and survival data were obtained for each experiment.

## RESULTS

A gross examination at post-mortem presented an interesting difference in the color of the musculature and in the gizzards of the birds on this experiment. Those fed the basal ration, basal ration and mild doses of desiccated thyroid were normal light-colored carcasses. The gizzards were well muscled while the cornified inner lining was smooth, tough and contained few ulcer-like lesions. The carcasses of the fluorine-fed birds without exception were dark in color and approached a cyanotic condition. The cornified lining of the gizzard was considerably roughened and contained many ulcerated lesions. It was quite fragile and broke easily when subjected to slight tension. In the case of lot III where the birds were injected with NaF solutions the meat was very dark and cyanotic resembling the appearance of the dark

TABLE 1  
*Summary of the daily ingestion of fluorine and desiccated thyroid in milligrams per kilogram of body weight by weekly periods*

WEEK LOT	1		2		3		4		5		6		7	
	F.	D.T.	F.	D.T.	F.	D.T.	F.	D.T.	F.	D.T.	F.	D.T.	F.	D.T.
Experiment 35A														
I	....	....	....	....	....	....	....	....	....	....	....	....	....	....
II	136.4	....	....	....	....	....	76.3	....	....	65.7	....	....	....	....
III	....	....	....	....	....	....	....	....	....	....	....	....	....	....
IV	110.5	221.0	....	184.8	....	....	120.2	240.4	....	....	....	....	....	....
V	118.6	474.0	....	....	....	....	....	....	....	....	....	....	....	....
VI	117.0	702.0	130.0	780.0	92.0	552.0	....	....	....	....	....	....	....	....
VII	....	612.0	....	483.0	....	436.0	....	343.0	....	....	350.0	....	....	....
VIII	....	678.0	....	683.0	....	565.0	....	636.0	....	....	554.0	....	....	....
Experiment 35B														
I	....	....	....	....	....	....	....	....	....	....	....	....	....	....
II	107.0	....	103.8	....	....	103.8	....	70.0	....	....	78.5	....	66.1	....
III	38.0	....	38.0	....	....	38.0	....	38.0	....	....	34.0	....	40.0	....
V	113.9	63.1	96.0	53.5	73.0	49.0	65.9	36.6	89.3	50.0	92.1	50.8	101.0	56.0
IV	96.6	106.0	102.2	113.0	98.0	109.0	100.0	111.0	97.6	108.0	90.0	95.9	88.9	98.6
VI	85.0	200.0	102.1	223.0	102.4	228.0	91.7	200.0	85.5	186.0	65.0	189.0	93.9	208.0
VII	....	216.0	....	220.8	....	199.0	....	128.0	....	235.0	....	179.0	....	187.0

meat from game birds. The cornified lining of the gizzards from these birds was, however, quite smooth, tough and showed no sign of the ulcers noted in the lots fed NaF.

A transitory paralysis of slight character appeared in nearly all birds fed 0.1 per cent, or more, of desiccated thyroid in combination with NaF. This condition was noted during the third and fourth weeks of the experiment. It automatically disappeared in the course of a few days. The paralysis was not observed to occur in the lots fed NaF or desiccated thyroid alone.

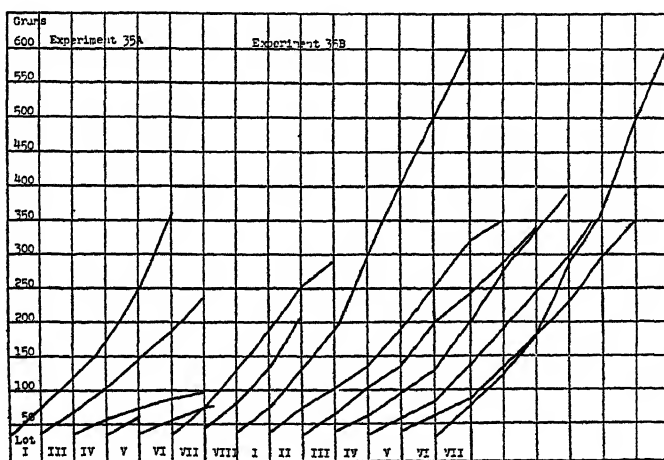


Figure 1

Inspection of table 1 indicates that the ingestion of fluorine ranged from 136 mg. of F per kilogram of body weight during the first week of the experiment to roughly 66 mg. per kilogram of body weight at the close of the experiment. Beyond the third week an average of 66 to 78 mg. of F per kilogram of body weight was ingested. This level of fluorine intake, or more, retarded growth as shown in figure 1. The effect upon growth seems to be a systemic reaction to fluorine. A tolerated level of F compatible with life was apparently reached and food consumption restricted to that level. Normally the feed consumption of growing chicks increases 300 per cent



in 5 weeks and 500 per cent in 7 weeks. The levels of fluorine fed in these studies restricted the increase in feed consumption to 150 per cent at 5 weeks and 300 per cent at the end of 7 weeks. Thus the growth stimulus was held in abeyance by the restriction of feed consumption coincident with the development of fluorosis. That the effect was systemic in nature was further supported by the reaction of lot III to NaF injections. Here too, the feed consumption dropped in the proportions noted when NaF was fed. This evidence indicates clearly that the restriction of feed consumption was not a direct reaction from, or associated with the digestive tract.

These experiments demonstrated the effects of various levels of desiccated thyroid upon the chick. Levels of 0.4 per cent and 0.6 per cent inhibited growth with the latter distinctly toxic. A level of 0.2 per cent had no inhibiting effect upon growth. An intake of 350 mg. of desiccated thyroid, or more, per kilogram of body weight retarded growth while approximately 200 mg. was without effect. Desiccated thyroid when fed alone failed to restrict feed consumption. The inhibition of growth in this case appears to be a more rapid utilization of metabolites thus producing its effect in a distinctly different manner from that of fluorine.

When NaF and thyroid were fed together the toxicity was markedly increased. Every combination of thyroid and NaF in experiment 35A was definitely more toxic than either substance alone. Growth was curtailed sharply. Table 2 indicates that these combinations were sufficiently toxic to produce collapse and death within a very short time. An intake of approximately 200 mg. of desiccated thyroid per kilogram of body weight which in itself was relatively harmless reduced the chick days survived to 60.5 per cent when fed with NaF. This was comparable to the highly toxic level of 0.6 per cent desiccated thyroid fed alone.

In experiment 35B an average daily intake of approximately 50 mg. of desiccated thyroid per kilogram of body weight proved ineffective in accentuating fluorine toxicity. When this level of ingestion was raised to a level of 100 or 200 mg.

per kilogram of body weight the survival period was lowered distinctly. It appeared that approximately 0.1 per cent of desiccated thyroid was necessary to augment the NaF action. At this level of feeding the intake of thyroid and fluorine were practically equal.

The injection of NaF at the rate of 64 or more mg. of F per kilogram of body weight produced rapid collapse characterized by relaxation and extension of the wings, drooped

TABLE 2  
*Survival data of chicks on experiment*

EXPERIMENT 35A				EXPERIMENT 35B			
Lot	Number of chicks	Total number of chick days	Days survived, per cent	Lot	Number of chicks	Total number of chick days	Days survived, per cent
I	6	210	100.0	I	10	490	100.0
II	6	186	88.5	II	10	490	100.0
III	6	...	....	III	10	316	64.5
IV	6	127	60.5	IV	10	490	100.0
V	6	50	23.8	V	10	370	75.5
VI	6	74	35.2	VI	10	337	68.7
VII	6	202	96.2	VII	10	433	88.4
VIII	6	104	49.5				

posture, hyperpnea followed by dyspnea, inactivity, rapid depression and death. Similar symptoms developed after each injection when doses sufficient to retard growth were given. Administration of NaF intraperitoneally was possible up to 40 mg. of F per kilogram of body weight. This level, as well as 16 mg., depressed growth significantly. Eight milligrams of desiccated thyroid per kilogram of body weight were without demonstrable effect upon growth. Similar levels of fluoride injection produced the same type of response in the older chicks.

## DISCUSSION

The observations made in this study are of considerable interest in view of the high fluorine tolerance of this species. We have considered that the tolerance of this species might be due to less complete or selective absorption from the digestive tract, a difference in the enzymatic systems involved in cellular metabolism, or to more effective elimination. It is apparent from intraperitoneal injections that the high tolerance to fluorine is not entirely due to incomplete or selective absorption. It appears likely that a combination of the other two factors operate to protect this species. However, the lower storage of fluorine in chronically poisoned hens, reported by Haman, Phillips and Halpin ('35), tends to indicate that more effective elimination plays an important role in the resistance of this species to chronic fluorine toxicosis.

An explanation for the interaction of desiccated thyroid and NaF which results in an augmentation of toxicity is not possible at this time. It seems likely that the results obtained with the chick resemble those with the rat in which the metabolic rate was increased by feeding desiccated thyroid and NaF (Phillips, English and Hart, '35).

## CONCLUSIONS

The high tolerance of the growing chick to NaF administration was again demonstrated. It appears that factors other than selective absorption are responsible for the fluorine tolerance of this species. Approximately 70 mg. of F per kilogram of body weight were necessary to inhibit growth in this species after the first week of life. This level of F intake inhibits growth through restriction of feed consumption. The intraperitoneal injection of NaF solutions likewise restricted feed consumption which indicates that the reaction to fluorine was systemic in nature and independent of any reaction via the digestive tract. The tolerance to intraperitoneal injections of NaF solution was distinctly lower, 64 mg. of F per kilogram of body weight were found to be lethal while 35 to 40 mg. per kilogram of body weight retarded growth.

The feeding of desiccated thyroid in amounts greater than 350 mg. per kilogram of body weight definitely retarded growth while levels of 200 to 225 mg. per kilogram of body weight were without effect. Feed consumption was not restricted by desiccated thyroid feeding and differs in this respect from fluorine toxicosis.

Non-toxic levels of desiccated thyroid were made distinctly toxic by chronic fluorosis induced by NaF feeding in the growing chick. Growth was greatly retarded and the survival period shortened. The feeding of the relatively harmless level of 0.2 per cent of desiccated thyroid with NaF produced results similar to that of the highly toxic level of 0.6 per cent desiccated thyroid. Thus fluorine ingestion enhances the toxicity of desiccated thyroid or vice versa.

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# MINIMUM VITAMIN A REQUIREMENTS WITH PARTICULAR REFERENCE TO CATTLE<sup>1</sup>

H. R. GUILBERT AND G. H. HART

*Division of Animal Husbandry, College of Agriculture, University of California, Davis*

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This paper continues the investigation previously reported (Guilbert and Hart, '34). It attempts to elucidate some quantitative vitamin A relationships that are important from practical and from scientific viewpoints. The data presented, although dealing primarily with cattle, so extend the quantitative knowledge concerning vitamin A requirements in widely varying species that some broad generalizations may be made concerning the minimum requirement of mammals.

## STORAGE OF VITAMIN A IN CATTLE

In the paper mentioned we showed that in cattle, as in other species, the vitamin A reserve of the newborn is low. The rate of storage, apparently, is relatively slow during the period of rapid growth and increases as the rate of growth decreases, so that maximum storage is obtained in the adult of advanced age. The data were presented in terms of relative values per unit weight of liver. As opportunity afforded, further data have been collected from animals of known history; and from these it has been possible to estimate the total vitamin A reserve in the body.

The carotene determinations were made by the colorimetric method on unsaponifiable fractions of liver and fat tissues,

<sup>1</sup> This report is part of an investigation on the relation of nutrition to reproduction which became cooperative with the United States Bureau of Animal Industry, July 1, 1929.

using the Sprague ('28) dye solutions standardized against beta carotene of high purity obtained through the courtesy of Dr. H. H. Strain, division of plant biology, Carnegie Institution of Washington, Stanford University. Since spectrographic equipment was not available, the vitamin A determinations were made upon the basis of the Carr and Price antimony trichloride reaction, the technic for which has been described (Guilbert and Hart, '34). One color unit by this technic is approximately the equivalent of the color produced by 1 microgram of carotene reacting with 2 cc. of saturated solution of antimony trichloride in chloroform. In the hands of a practiced technician, the variation in judging this end point is about  $\pm 10$  per cent. According to Moore ('33) and others, vitamin A gives approximately ten times as much color with antimony trichloride reagent as does an equivalent amount of carotene. The carotene content and total blue units were determined separately for both liver and adipose tissues. The number of blue units attributable to carotene were deducted from the total blue units; and the remainder, divided by the factor 10, gave a rough estimate of the number of micrograms of vitamin A present. Carotene and total blue-unit determinations were made on both abdominal and back fat, and the mean values were used in the calculations. The total fat in the body was estimated by the formulae of Lusk ('26). Liver weight, when not obtained, was calculated as 1 per cent of body weight. Swett, Graves and Miller ('28) have shown that in very fat beef animals the liver weight may be less than 1 per cent of the live weight, while Swett, Miller, Graves and Mathews ('33) in their extensive data on dairy cattle have found that the liver weight averages about 1.3 per cent of the live weight. Estimating the liver weight, therefore, does not introduce serious errors for the present purpose. In cattle whose livers had 300 to 500 blue units of vitamin A per gram, the lung and spleen extracts gave negative tests for vitamin A, and the kidneys contained only 1 to 5 units per gram. The amount of vitamin A reserve in tissues other than fat, liver and blood serum may be con-

sidered negligible. Semb, Baumann and Steenbock ('34) presented data suggesting that the blood stream of cattle may function as a significant storage reserve for carotene—a source not considered in the data presented in table 1.

Animals nos. 27, 32, 29 and 18 had been used in an experiment on phosphorus deficiency as influencing energy utilization. Nos. 27 and 32 were fed low phosphorus rations, whereas nos. 29 and 18 were the controls. All were placed on experiment at about 1 year of age. Before this time they had access to green feed constantly. During the first 22 months of the experimental period the vitamin A was furnished by 0.9 to 1.4 kg. of fair quality field-cured alfalfa meal and about 30 cc. of cod liver oil daily. During the last 8 months the alfalfa was omitted, and 30 cc. of cod liver oil daily constituted practically the sole source of vitamin A. The feed consumption of these animals was low, especially for those receiving inadequate phosphorus. During the last 8 months the rations contained no roughage. The livers were below average in weight. Although the cod liver oil used was biologically tested by the manufacturer, a later antimony trichloride test showed that it had comparatively low potency. The final reserves of the animals were probably lower than at the beginning of the experiment.

The remainder of the animals were from the university beef herd, and all had had access to green feed and alfalfa hay in abundance throughout their lives. Nos. 26 and 275 had finished lactation periods shortly before being slaughtered. No. 96 had not lactated for about a year. Her liver storage represented the highest values obtained from old cows that have had ample opportunity for storage throughout their lives.

#### DISCUSSION

According to the data for the first four animals in table 1, the tissues were almost carotenoid-free, and the total reserve was significantly lower than in younger animals having access to abundant green feed. The reserves in the 2-year-old heifers (nos. 469 and 448) were much lower per unit weight of liver



and in total amount than those in the adult cows. The proportion of the total vitamin A reserve found in the body fat varied from 7 to 33 per cent. In the normally fed animals the liver reserve was largely vitamin A, whereas in the fat tissue the blue value obtained only slightly exceeded that attributable to the carotene content, showing that this part of the reserve existed largely as carotene.

TABLE 1  
*Storage of vitamin A and carotene*

ANIMAL NO.	AGE, YEARS	LIVE WEIGHT, KILOGRAMS	LIVER WEIGHT, KILOGRAMS	WEIGHT OF BODY FAT, KILOGRAMS	CAROTENE, MICROGRAMS PER GRAM		VITAMIN A, MICROGRAMS PER GRAM		CAROTENE AND AND VITAMIN A IN MILLIGRAMS		
					Liver	Body fat	Liver	Body fat	Liver	Body fat	Total
27	3½	388	3.05	80 <sup>1</sup>	Trace	Trace	31	0.15	95	12	107
32	3½	353	2.77	70 <sup>1</sup>	Trace	Trace	35	0.33	97	23	120
29	3½	525	3.27	130 <sup>1</sup>	Trace	Trace	42	0.15	137	20	157
18	3½	530	3.60	135 <sup>1</sup>	Trace	Trace	50	0.10	180	14	194
469	2	480	4.80 <sup>2</sup>	100 <sup>3</sup>	3.5	1.7	100	0.60	497	230	727
448	2	523	5.20 <sup>2</sup>	105 <sup>3</sup>	3.0	1.0	80	0.40	432	147	579
275	6	620	6.20 <sup>2</sup>	176 <sup>3</sup>	12.0	1.9	200	0.50	1315	422	1737
26	12	566	5.60 <sup>2</sup>	85 <sup>3</sup>	8.0	6.5	200	0.30	1165	577	1742
96	18	590	5.90 <sup>2</sup>	164 <sup>3</sup>	6.5	5.2	450	0.30	2693	902	3592

<sup>1</sup> Computed by the formula of Lusk ('26). Percentage of fat in entire animal =  $9.073$  (per cent of caul fat to live weight) +  $0.936$  (dressing per cent) —  $44.96$  per cent.

<sup>2</sup> Estimated as 1 per cent of the body weight.

<sup>3</sup> Estimated by the formula of Lusk ('26). Per cent of fat in entire animal =  $1.782 \times$  dressing per cent —  $86.4$  per cent.

About 225 days were required to deplete the vitamin A reserves of animals whose controls were autopsied and found to have liver storage similar to that of nos. 469 and 448. If it is assumed that the reserves of these animals (nos. 469 and 448) including an average amount in the blood serum, would suffice for a similar period, the indicated withdrawal from storage is 4 to 5 mg. daily, or about 9 to 11 micrograms per kilogram live weight daily.

Because of the limitations in accuracy of the vitamin A determination and the calculations involved, these figures are

admittedly open to criticism; but we present them because they give greater insight into quantitative relationships with regard to cattle than has been obtained heretofore. Baumann, Riising and Steenbock ('34 a) have presented data showing that the rate of depletion of vitamin A stores in rats decreased as the depletion advanced, that is, as the reserves became smaller. Our data on cattle, although limited to a few animals, showed the same tendency. Furthermore, a daily allowance of carotene approximating the minimum requirements for health and normal gain, when added to a carotenoid-deficient basal ration, had little or no sparing action on the rate of loss from storage (Guilbert and Hart, '34). As Baumann, Riising and Steenbock ('34) have shown, the daily intake of vitamin A must considerably exceed the minimum requirement for normal growth before storage occurs. Evidently the intake must be in considerable excess of daily requirement either to maintain already existing reserves or to provide for storage in depleted animals. The estimation of minimum requirement by studying the rate of depletion of reserves, therefore, has limitations. On the other hand the estimate of 9 to 11 micrograms per kilogram live weight appears significant when considered along with the direct determination of carotene requirement of depleted cattle discussed in the next section of this paper.

Young pasture grass or fresh green alfalfa ordinarily contains 35 to 50 mg. of carotene per 100 gm. of dry matter. About 10 kg. of dry matter, therefore, would furnish 3.5 to 5.0 gm. of carotene—an amount equivalent to the estimated maximum storage in a very fat, aged cow that had had access to green feed in abundance throughout her life.

The admission of relatively large errors in the estimations of storage would not detract from the conclusion that cattle ingest in a few days' grazing on green forage more carotene than they store (as carotene and vitamin A) in a lifetime. The fact that during the first 2 or 3 years of life the concentration of reserves in the tissues does not nearly approach that found in aged cattle would indicate that the rate of storage is slow. A depleted cow, no. 440, however, fed about 15

gm. of carotene in freshly cut alfalfa during a 13-day period, stored approximately 400 mg. or 2.7 per cent of that ingested—an average of 30 mg. daily in excess of her daily requirement. The concentration in the liver of 500 blue units per gram was similar to that of 1- to 2-year-old cattle kept under optimum conditions. This fact suggests that storage may be comparatively rapid until a certain level is reached but that further concentration in the tissues may proceed more slowly. The percentage recovery by this cow compares favorably with the recovery of carotene by rats, as reported by Davies and Moore ('34).

#### MINIMUM CAROTENE REQUIREMENT OF CATTLE

In a study of the effect of various nutritional deficiencies in beef cattle during gestation and lactation, a number of animals were depleted of their vitamin A reserves. During the earlier investigations, animals receiving 1 pound daily of a fairly green chopped alfalfa hay in addition to vitamin A low basal rations showed no clinical symptoms of deficiency after a period 7 months longer than was required to deplete similar animals on the basal ration alone. Two of these animals exhibited no symptoms even though their milk was so deficient in vitamin A that their nursing calves developed definite night blindness and other deficiency symptoms. Thus we had fed empirically an amount of hay with carotene content close to the minimum to prevent the onset of deficiency symptoms. This was the starting point for study of minimum requirement by the curative method with ten experimental animals, nos. 410, 426, 429, 434, 438, 440, G54, 541, 542 and 543.

The concentrate mixtures used in the various basal rations given these animals are shown in table 2.

The carotene content of the concentrate mixtures was less than 0.02 mg. per cent. For roughage, well-bleached wheat or barley straw was used with only a trace of carotene in the extract from 100 gm. Thus the carotene in the basal rations was practically negligible.

The carotene determinations on the alfalfa supplement were made colorimetrically, using the technic described by Guilbert ('35). From November, 1934, the dehydrated alfalfa meal substituted for chopped alfalfa hay was kept in a refrigerator at about 5°C., a temperature which practically eliminates deterioration in carotene content. Samples for analysis were collected as the individual feeds were being weighed into paper bags for each animal. Sufficient feeds were weighed at one time to last 1 to 2 weeks. Carotene determinations on the composite samples were made at monthly intervals. The

TABLE 2  
*Concentrate mixtures used in various basal rations*

FEEDS	CONCENTRATE MIXTURE NO.				
	1	2	3	4	5
	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>	<i>Per cent</i>
Dried molasses beet pulp	99.0	98.0	84.0	70.0	
Rolled barley				14.0	34.5
Rolled oats					27.0
Wheat bran					27.0
Cottonseed meal			14.5	15.0	10.0
CaCO <sub>3</sub>	1.0		0.5	1.0	1.5
Mono-calcium phosphate or decarbonized bone black		2.0	1.0		
Per cent digestible crude protein	5.0	5.0	9.5	10.0	12.7
Per cent calcium	1.0	0.9	0.9	0.9	0.7
Per cent phosphorus	0.06	0.4	0.4	0.3	0.7

samples were kept in sealed jars and were stored at a temperature of about —5°C. until analysed.

The nine animals used varied in age from 7 months to 4 years and in weight from 130 to 500 kg. They were fed individually and had the run of paved corrals with access to direct sunlight except at feeding time.

Recovery experiments were started when the animals exhibited complete blindness in semi-darkness. In most cases they also showed nervous symptoms (convulsions). Starting with small amounts of chopped alfalfa hay or dehydrated alfalfa meal of known carotene content, the dose was increased

at intervals until the amount ingested was sufficient to provide for normal weight increases and to cure all clinical symptoms of deficiency. Sub-optimum levels of carotene intake were attained that permitted nearly normal growth yet night blindness persisted. The level of carotene intake at which night blindness disappeared permitted gains up to 1.3 kg. daily. The presence or absence of night blindness, the first detectable clinical symptom of deficiency, therefore constituted a delicate index upon which minimum requirement could be based. All animals were tested for night blindness at frequent intervals by driving them about the corrals in twilight, moonlight, or dim electric light. By this means the normal and deficient animals were readily detected.

The minimum carotene requirement was established by repeatedly increasing or decreasing the intake of alfalfa supplement by each animal until night blindness and other symptoms disappeared or reappeared. The chopped alfalfa hay used varied in carotene content from 2.1 to 3.3 mg. per cent, while the dehydrated alfalfa meal varied from 11.6 to 18.9 mg. per cent. The response in all cases was proportional to the amount of carotene ingested. To obtain further evidence relative to the possibility of factors other than vitamin A being involved, three animals exhibiting deficiency symptoms were given either halibut or cod liver oil. All clinical symptoms disappeared in the same manner as when alfalfa was fed and weight increases were resumed.

After 2 years of experiment with these animals involving long growth periods with the younger animals and the entire gestation period of one of the cows it was evident that the daily minimum requirement of carotene as supplied by alfalfa lay between 26 and 33 micrograms per kilogram live weight. In most instances symptoms recurred when the level of carotene intake fell below 29 micrograms per kilogram live weight. Symptoms regularly reappeared within 2 weeks after the supplement was withdrawn from the ration of animals that had been receiving the minimum level, showing that very little storage occurred.

Three animals were finally allowed to reach advanced stages of deficiency involving total blindness, frequent convulsions, diarrhea, failure of appetite, emaciation and in two of the cases clouding of the corneas. One of the animals died during a convulsion and a histological examination was made of some of the tissues. The remaining two animals were given vitamin A supplements. One of these, a bull, was given two subcutaneous injections of 100 mg. each of carotene dissolved in olive oil, at intervals of 2 weeks. A corneal lesion disappeared in 5 days and slight improvement in physical condition occurred, but there was no weight increase during a period of 1 month. At the end of this time 0.5 cc. of halibut liver oil was administered daily, and was followed by remarkable response both in weight increase and improvement in physical appearance. After 30 days alfalfa was substituted for the liver oil and improvement continued. The animal remained permanently blind. It fully recovered in other respects, was an active breeder and the semen contained motile sperm. The other animal, a heifer, recovered nearly normal vision in daylight, but still shows after several months, defective vision in twilight, regardless of the amount of vitamin A or carotene fed, indicating an impairment of a permanent nature as contrasted with simple night blindness.

#### DISCUSSION

*Minimum requirement.* From the above data the daily minimum carotene requirement of cattle is 26 to 33 micrograms per kilogram live weight. Carotene intake at the level of about 29 micrograms per kilogram live weight prevented or cured clinical symptoms of deficiency and permitted normal weight increases when the basal ration was otherwise adequate and not restricted in amount. When the carotene intake fell below this level, clinical symptoms reappeared simultaneously with cessation or reduction in weight increases. No detectable difference in response occurred when the same level of carotene intake was attained by feeding either a field-cured hay or about one-tenth this quantity of dehydrated meal. Cod liver

oil or halibut liver oil produced the same response in deficient animals as alfalfa.

The minimum daily requirement of 26 to 33 micrograms of carotene per kilogram live weight, when furnished by alfalfa, is significantly close to the estimated daily withdrawal of reserves from storage of 9 to 11 micrograms per kilogram live weight, considering the amount of decomposition of ingested carotene that is liable to occur in the digestive tract.

Since the minimum level of carotene intake appeared to be the same per unit of body weight in cattle varying from about 130 kg. to 500 kg., the question arises whether or not vitamin A requirement is related to the amount of body substance or to energy requirement. The differences in body size are not sufficient to give convincing evidence on this point from the cattle data alone. In order to examine this question further, data on species varying in size from the rat to the cow have been assembled in table 3. Comparable data were used with reference to minimum requirement for growth approaching normal and the prevention of clinical symptoms.

When the data are calculated to micrograms of carotene or vitamin A per kilogram body weight, the agreement is exceedingly good in mammals varying in size from 100-gm. rats to 500-kg. cows. Dunlop's data ('34) on swine have rather wide limits: the low level sufficed for nearly normal gains, but deficiency symptoms persisted; the high level exceeded minimum requirement and provided for considerable storage. The minimum requirement, therefore, was probably closer to 13 micrograms per kilogram than to 60 micrograms per kilogram. The mean is, however, fairly close to the values for the rat and the cow.

If the metabolism of rats and cattle were proportional to the body surfaces or the two-thirds power of the body weight then the metabolism per unit of body weight should be 17.1 times greater in 100-gm. rats than in 500 kg. cows. If the metabolism is proportional to the three-fourths power of the body weight (Kleiber, '32) the rat should have a metabolic rate 8.4 times that of the cow. To be proportional to energy

TABLE 8  
*Daily vitamin A and carotene requirements per unit of body weight of different species*

SPECIES	SOURCE OF DATA	APPROXIMATE WEIGHT OF ANIMALS, KILOGRAMS	DAILY INTAKE, MICROGRAMS		GAIN	CAROTENE OR VITAMIN A INTAKE DAILY PER KILO- GRAM OF BODY WEIGHT, MICROGRAMS
			Vitamin A	Carotene		
Rat	Moore ('33)	0.150	3.0	3.0	Good	20.0
Rat	Coward ('30)	0.100	2.4 <sup>1</sup>	.....	Good	24.0
Rat	Baumann et al. ('34 b)	0.115		2.8	Good	25.0
Swine	Dunlop ('34)	45.000		600.0 <sup>2</sup>	Good	13.0
Swine	Dunlop ('34)	45.000		2660.0 <sup>3</sup>	Good	60.0
Cattle	Authors data	130.000-500.000		3400.0-16500.0	Good	26.0-33.0 (from alfalfa)
Chicken	Kline et al. ('32)	0.400		30.0	Good	75.0 (carotene in oil)
Chicken	Guilbert and Hinshaw ('34)	0.680		190.0 <sup>4</sup>	Good	280.0 (2% alfalfa meal)
Chicken	Guilbert and Hinshaw ('34)	0.680		380.0 <sup>4</sup>	Good	560.0 (4% alfalfa meal)
Turkey	Guilbert and Hinshaw ('34)	1.750		468.0 <sup>5</sup>	Moderate	270.0 (4% alfalfa meal)
Turkey	Guilbert and Hinshaw ('34)	2.570		1600.0 <sup>6</sup>	Good	620.0 (8% alfalfa meal)

<sup>1</sup> Estimated on the basis that 0.3  $\gamma$  produces the minimum response (1 unit) and that approximately eight times the minimum produces a gain of about 10 gm. weekly, as shown by the curve of response (Coward, '30).

<sup>2</sup> Dunlop ('34) states: "The vitamin A requirement of swine has been shown to lie between 14 and 62 mg. of carotene per 100 pounds of ration." A daily food intake of 4.3 pounds for 100 pounds body weight (the schedule used by Dunlop) was used in making this calculation. The lower level supported nearly normal growth, but deficiency symptoms persisted; on the high level considerable storage occurred.

<sup>3</sup> The carotene intake was about minimum; the amount of storage was small.

<sup>4</sup> The carotene intake exceeded requirements and provided for considerable storage.

<sup>5</sup> The level of carotene intake from 4 per cent of dehydrated alfalfa meal produced slightly less than normal growth with slight storage in surviving birds. There was some mortality with A avitaminosis symptoms.

<sup>6</sup> The carotene intake provided by 8 per cent dehydrated alfalfa meal was adequate for normal growth and a significant amount of storage.



requirement, therefore, the vitamin A requirement per unit of body weight would have to be from eight to seventeen times greater in the 100-gm. rat than in the 500-kg. cow. Since, however, the requirement per kilogram of body weight is practically the same for species varying so much in size and in other characteristics as the rat, the pig, and the cow, we feel justified in advancing the hypothesis that the vitamin A requirement is proportional to body weight and that in mammals it is in the order of 20 to 30 micrograms per kilogram of body weight. Confirmation of this generalization has been obtained in experiments at this station with swine (Hughes and Guilbert, unpublished data) and with sheep (Miller and Guilbert, unpublished data).

If vitamin A requirement is directly proportional to body weight while energy intake is proportional to the three-fourths (or two-thirds) power of the body weight, then, other factors being equal, the percentage of vitamin A in the ration must be increased as body size increases to compensate for the decrease in food consumption per unit of body weight. Some of the errors and difficulties encountered in the past in applying data from one species to another may be explained on this basis. The general impression that young, rapidly growing animals have a higher vitamin A requirement than adults is explained largely by the fact that young animals are depleted sooner than older ones on the same deficient diet. This can be explained on the basis of lower reserves in the young animal and 'dilution' of the reserve as body size increases.

As shown in table 3, the minimum requirement of chickens and turkeys per kilogram of body weight appears to be much higher than that of the mammals studied. In this connection the data of Kline, Schultz and Hart ('32) are especially valuable. Birds receiving daily 30 micrograms of carotene (dissolved in cottonseed oil) gained normally until they attained 400 gm. in weight, then the allowance became inadequate. The experiments of Guilbert and Hinshaw ('34) offer further evidence that the requirement is related to body weight rather

than to total food consumption. Growth, liver storage data and clinical observation all showed that adding 2 and 4 per cent of dehydrated alfalfa meal to an otherwise vitamin A deficient ration fed to chickens gave results comparable to those obtained by adding 4 and 8 per cent, respectively to the same basal ration fed to turkeys. The food consumption of the chickens per unit of body weight, however, was approximately double that of the turkeys, so that the carotene intake per kilogram of body weight was in good agreement, as shown in table 3. The carotene intake by chickens on the 2 per cent alfalfa meal level was slightly more per unit of body weight than that of the turkeys on the 4 per cent level. Symptoms of deficiency developed in some of the turkeys but not in the chickens. This level in chickens appeared close to the minimum and indicates that the availability of carotene in alfalfa for this species is not so high as the availability of carotene in oil.

*Requirement for reproduction.* The evidence on the minimum requirement for reproduction may be summarized as follows: Five females began their gestation periods with normal storage and were limited to a daily allowance of 9.5 to 15 mg. of carotene, furnished by 1 to 1.5 pounds of alfalfa hay. As has been shown previously, this amount does not prevent storage losses from occurring at about the same rate as when none is fed, at least during the first half of the depletion period. Six calves were produced by these animals. Two successive calves from no. 438 developed diarrhea and died during the first week. Two calves from nos. 410 and G 54, respectively, developed diarrhea, became very weak, but recovered after cod liver oil therapy; one calf from no. 440 recovered from severe diarrhea during the first week without treatment; and one calf from no. 434 was practically normal. The liver of one calf that died contained a trace of vitamin A; the liver of the other contained none. Evidently the mothers' reserves were low, and a carotene intake at approximately the minimum level for the mother was insufficient for the production of normal calves. In contrast to these animals, heifer

no. 426 began her gestation period with no reserve of vitamin A. The daily intake of carotene for the first 8 months of pregnancy (during which she was not lactating) averaged about 30 micrograms per kilogram live weight. During the ninth month the amount was increased to a little more than three times the minimum (90 micrograms per kilogram live weight), and a normal calf was produced. It showed no symptoms of deficiency the first 2 months of nursing, during which the daily carotene intake of the mother was at the level of 143 micrograms per kilogram live weight.

The minimum carotene requirement for the production of milk containing optimal amounts of vitamin A and carotene according to the data of Semb, Baumann and Steenbock ('34) greatly exceeds the animals' minimum requirement plus the amount recovered in the milk.

Nos. 426 and 410 came into oestrus during the time they exhibited night blindness and convulsions. No. 410 accepted service but did not conceive.

*Clinical symptoms.* Night blindness precedes nervous symptoms by one to several weeks. In the early stages, convulsive seizures were sudden, the animal falling as though stunned. Later the animals appeared to feel the attack coming on and braced themselves against falling, swaying from side to side before going down. The attacks lasted from one to several minutes. Total blindness was commonly the next stage and involved, presumably, degenerative changes in the optic nerves. The pupils became widely dilated. At this time a rather characteristic greenish color from the retina is manifested when looking through the cornea with the animal facing the light. Ophthalmoscopic examination made at night, showed this change more distinctly when compared with the yellowish color of the retina in normal animals. Clouding of the cornea appeared, in our experience, in the later stages of deficiency. The onset of symptoms may vary somewhat in different individuals and various localized paralyses may occur.

Blindness at birth in the second calf from cow no. 429 is interesting in the light of previous publications describing

similar conditions. This cow was on minimum vitamin A during her first gestation period, the intake being limited to that contained in 1 pound of alfalfa hay daily from November 23, 1932 to September 26, 1933. On the latter date she was bred, and the alfalfa hay was removed from her diet. She definitely exhibited night blindness on March 6, 1934, in the fifth month of pregnancy. From this time on, ample vitamin A was supplied to ascertain what affect this recognizable deficiency early in pregnancy might have on the offspring.

Crocker ('19) reported blindness caused in newborn calves from what he termed insidious rachitis. There were sixteen cases in Guernseys on two farms in Pennsylvania. No external change was present in the eyes. The dams were all on high concentrate and low roughage feeding for intensive production. On post-mortem the affected calves showed stenosis of the optic canal with chronic optic neuritis, and the condition was diagnosed as insidious rachitis with deformation of the sphenoid bone.

de Schweinitz ('31) and de Schweinitz and De Long ('34) have published on a similar condition in calves from Guernsey cows under the name of papilledema or choked disc, with the suggestion that the defect may be hereditary. A paper by Moore, Huffman and Duncan ('35) describes twenty-four cases in calves and growing dairy animals, of a type of nutritional blindness which the authors state apparently differs from true vitamin A blindness. In their cases blindness without inflammation of the external eye structure was associated with weakness, spasms, and paralysis. We have considered this syndrome in our experimental animals to be characteristic of vitamin A deficiency in cattle, although we have seen marked ulceration of the cornea in some naturally occurring cases (Hart and Guilbert, '33) and in some of the animals in the experiments of Mead and Regan ('31).

Degeneration of nerve tissue, including that of the optic nerve, is well established in vitamin A deficiency. All the cases reported by the workers mentioned above appear to us to be manifestations of vitamin A deficiency. Since the defective sight can be corrected if vitamin A therapy is started

in time, evidently the stenosis of the optic canal is secondary to degenerative changes and to atrophy of the optic nerve.

One case of edema of the legs similar to those reported by Bechdel, Honeywell and Dutcher ('28) was observed. It is suggested that the edema may be concerned with renal involvement. Direct proof, however, is lacking, for the total non-protein nitrogen of the blood serum was normal in our case. No urine examination was made.

Degenerative changes in the kidneys of cattle appear to be common in advanced deficiency. No sperm were found in the testes of bull no. 541 that died of vitamin A deficiency. Although the microscopic picture of sections of testicle and kidney may have been complicated by post-mortem changes, there was good evidence of degenerative alterations attributable to deficiency.

#### SUMMARY

The total storage of vitamin A and carotene in the liver and body fat of cows 2 to 18 years old, which had access to green feed in abundance throughout life, was estimated to be about 0.6 to 0.7 gm. in the younger animals and up to 3.6 gm. in aged cows. From 67 to 93 per cent of the storage was in the liver. The amount in other organs was negligible. In the liver most of the storage was in the form of vitamin A, whereas in the fat, carotene predominated.

A comparison between the estimated total storage, including that in the blood stream, and the time required to deplete similar animals on a carotenoid-deficient ration, indicated a daily withdrawal of 9 to 11 micrograms per kilogram live weight.

Cattle may ingest more carotene in a few days' grazing on green pasture than is stored in the body of a very fat old cow that has had continual access to green feed or alfalfa hay. A cow whose reserves were depleted stored about 400 mg. of carotene in a 13-day period, during which she ingested 240 kg. of freshly cut alfalfa containing about 15 gm. of carotene. Although the percentage recovery was low (2.7

per cent), a relatively rapid storage was shown. The concentration of the reserves of young animals as compared with that of aged adults, however, suggests that reserves above a certain level are accumulated slowly.

Subcutaneous injections of carotene, dissolved in olive oil, into a vitamin A deficient calf were followed by disappearance of a corneal lesion and by slight improvement in physical condition; but gain in weight did not occur. Apparently the injected carotene was slowly absorbed or poorly utilized.

Night blindness was found to be the first detectable clinical symptom of vitamin A deficiency and to constitute a delicate index upon which minimum requirements could be based. The progressive development of other symptoms of deficiency is presented in the discussion.

The daily minimum carotene requirement of the bovine is 26 to 33 micrograms per kilogram live weight. Carotene intake at the level of 29 micrograms per kilogram daily prevented or cured clinical symptoms, including night blindness, and promoted normal weight increases; yet it resulted in no storage. When the intake fell below this level, night blindness reappeared, and gains decreased.

Normal reproduction occurred in one instance in a cow that began gestation with no reserve and whose carotene intake was maintained at about the minimum level until the last month of pregnancy, when it was increased about threefold.

Vitamin A deficiency that had progressed to the point of night blindness and convulsions did not inhibit the occurrence of oestrus.

From a study of the minimum requirement to prevent or cure symptoms of deficiency and to permit growth at approximately the normal rate in rats, swine, sheep, and cattle, the hypothesis is advanced that vitamin A requirement is related to body weight rather than to energy requirement and that the minimum requirement of mammals is in the order of 20 to 30 micrograms of vitamin A or carotene daily per kilogram of body weight. This implies that, to compensate for lower food consumption per unit of weight, large animals require

a higher percentage of vitamin A in the diet than do small animals.

The carotene requirement per unit of body weight of chickens and turkeys appears to be equal, but the requirement of both species is considerably higher than that of the mammals studied, thus indicating a difference between birds and mammals in this regard.

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# IS THE WORK OF THE KIDNEY, DUE TO THE EXCRETION OF UREA, A FACTOR IN SPECIFIC DYNAMIC ACTION?

A. G. EATON, SHIRLEY C. CORDILL AND J. L. GOUAUX

WITH THE TECHNICAL ASSISTANCE OF VERA CLAY

*Department of Physiology, Louisiana State University Medical Center, New Orleans*

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That the kidney performs work in the concentration of the urine cannot be denied. This has been well proved by the experiments of Barcroft and Brodie ('05, '06) and by Hayman and Schmidt ('27-'28) by means of studies of the blood gases and by Tangl ('11, '13) who studied the energy metabolism of dogs before and after complete nephrectomy. Tangl concluded that the energy production of the kidneys was equal to about 8 per cent of the total metabolism. In this paper, we are concerned chiefly with the work of the kidney as a possible cause of the specific dynamic action of proteins.

Tangl ('11) administered 10 and 12 gm. of urea to dogs by stomach and obtained only a slight rise in metabolism. However, in one dog, the intravenous injection of urea caused a considerable increase in oxygen consumption (27 per cent). Lusk ('12) concluded from two experiments on a dog that the administration of urea by stomach tube caused no increase in heat production. These results were confirmed by Grafe ('16). Lublin ('28) reinvestigated the subject and reported an increase in metabolism not only when he administered urea, but also when he secured diuresis by giving large amounts of water by mouth. In his experiments the increased heat production was usually coincident with the greatest diuresis instead of occurring during concentration of the urine.

Borsook and Winegarden ('31 a, b, c) after a mathematical study of many of the important papers concerning the specific dynamic action of proteins came to the conclusion that 25 to 60 per cent of the specific dynamic action of proteins is due to the work of the kidney, that the energy consumed by the kidney is 6 to 11 Cal. per gram of nitrogen excreted and that the efficiency of the kidney is probably not more than 1 to 2 per cent. They also report on experiments carried out using students as subjects. The subjects were apparently untrained and a clinical type of apparatus was used for the metabolic determinations.

#### METHODS

The three authors and a departmental technician served as subjects for this series of experiments. None of them showed any kidney disease. All were thoroughly accustomed to metabolic determinations, having served as subjects for previous experiments. A light dinner was eaten the night previous to the test, a good sleep of 8 hours or more was obtained and no food or beverage, except water, was consumed until the completion of the experiment. Most of the experimental period was spent resting quietly on the metabolism cot.

One basal determination was made each morning after which the urea solution was drunk. In most cases 30 gm. of urea were given and any exceptions are noted in the table. Four further metabolic determinations were made during the successive 4 hours. Control days, when no urea was taken, were interposed.

The air samples were collected in a Tissot spirometer and analyses of this expired air were made and carefully checked by means of a modified Haldane analyzer accurate to 0.005 per cent. All analyses were made by the same individual. The accuracy of the apparatus was checked by frequent analyses of outdoor air.

Urine samples were collected hourly unless otherwise noted in the table. Each sample was measured and the specific gravity estimated by means of a urinometer. Nitrogen determinations were carried out in duplicate by the usual macro Kjeldahl method.

TABLE 1  
Subject I, white female

6/19/34					
	Cal./hr.	cc. urine/hr.	Sp. gr.	Per cent $N_2$	Gm. $N_2$ /hr.
Basal	54.2	289	1.004	0.081	0.234
10 gm. urea in 400 cc. warm water and 400 cc. water through day					
1st hour	54.4	316	1.004	0.235	0.739
2nd hour	54.3	274 <sup>1</sup>	1.005	0.218	0.597
3rd hour	53.8	274 <sup>1</sup>	1.005	0.218	0.597
4th hour	54.0	487	1.002	0.059	0.287
Total change — 0.3 Cal.					

<sup>1</sup> Collected and analyzed together.

7/10/34					
Basal	54.2	300	1.002	0.081	0.243
20 gm. urea in 400 cc. warm water and 400 cc. water through day					
1st hour	54.0	353	1.002	0.207	0.731
2nd hour	51.0	485	1.003	0.206	0.999
3rd hour	51.8	262	1.004	0.361	0.946
4th hour	51.3	590	1.001	0.146	0.861
Total change — 6.7 Cal.					

1/9/35					
Basal	56.0	175	1.006	0.229	0.401
400 cc. warm water, no urea					
1st hour	56.4	378	1.001	0.168	0.635
2nd hour	53.6	162	1.005	0.224	0.363
3rd hour	54.6	198	1.001	0.253	0.501
4th hour	52.1	48	1.011	0.476	0.228
Total change — 7.7 Cal.					

1/14/35					
Basal	52.7	289	1.005	0.143	0.413
400 cc. warm water and 400 cc. warm water through day, no urea					
1st hour	53.7	143	1.005	0.188	0.269
2nd hour	52.9	147	1.003	0.165	0.243
3rd hour	52.7	309	1.002	0.121	0.374
4th hour	55.4	105	1.004	0.269	0.282
Total change + 3.9 Cal.					

1/18/35					
Basal	54.2	366	1.003	0.162	0.593
30 gm. urea and approximately 600 cc. warm water					
1st hour	53.7	324	....	0.540	1.750
2nd hour	53.8	166	1.013	1.123	1.864
3rd hour	53.4	116	1.015	0.930	1.079
Approximately 500 cc. warm water					
4th hour	54.9	227	1.004	0.437	0.992
Total change — 1.0 Cal.					

TABLE 1—*Continued*  
*Subject II, white female*

	Cal./hr.	cc. urine/hr.	Sp. gr.	Per cent $N_2$	Gm. $N_2$ /hr.
1/23/35					
Basal	48.7	22	1.026	1.260	0.277
Approximately 600 cc. water and approximately 400 cc. water through day, no urea					
1st hour	53.9	90	1.009	0.538	0.484
2nd hour	52.6	47	....	0.694	0.326
3rd hour	53.1	29	....	0.980	0.284
4th hour	52.4	229	1.004	0.227	0.520
Total change + 17.2 Cal.					
1/28/35					
Basal	51.4	21	....	1.565	0.329
30 gm. urea with approximately 1000 cc. water					
1st hour	50.9	200	1.012	0.787	1.574
2nd hour	55.6	164	1.013	1.140	2.312
3rd hour	49.5	112	1.014	1.378	1.543
4th hour	49.5	107	1.017	1.442	1.543
Total change — 0.5 Cal.					
2/6/35					
Basal	51.7	61	1.008	0.336	0.205
30 gm. urea with approximately 600 cc. water					
1st hour	49.4	440	1.003	0.319	1.404
2nd hour	52.8	227	1.010	0.675	1.532
3rd hour	51.7	170	1.013	0.973	1.654
4th hour	49.8	160	1.013	1.002	1.603
Total change — 3.1 Cal.					
2/13/35					
Basal	51.7	275	1.002	0.085	0.234
30 gm. urea with approximately 600 cc. water					
1st hour	51.1	350	1.004	0.332	1.162
2nd hour	52.0	221	1.013	0.973	2.150
3rd hour	48.9	204	1.011	1.025	2.091
4th hour	54.3	384	1.008	0.449	1.724
Total change — 0.5 Cal.					
..					
<i>Subject III, white male</i>					
6/21/34					
Basal	65.1	144	1.004	0.224	0.323
20 gm. urea in approximately 500 cc. water					
1st hour	67.3	512	1.003	0.218	1.116
2nd hour	64.5	485	1.003	0.280	1.358
3rd hour	64.5	416	1.002	0.246	1.023
4th hour	64.0	423	....	0.246	1.041
Total change — 0.1 Cal.					
6/29/35					
Basal	61.2	139	1.006	0.370	0.514
20 gm. urea in approximately 800 cc. water					
1st hour	63.7	372	1.003	0.302	1.123
2nd hour	63.6	108	1.004	0.633	1.139
3rd hour	60.9	225	1.005	0.546	1.229
4th hour	60.1	300	1.003	0.339	1.017
Total change + 3.5 Cal.					

TABLE 1—Continued

1/15/35					
	<i>Cal./hr.</i>	<i>cc. urine/hr.</i>	<i>Sp. gr.</i>	<i>Per cent N<sub>2</sub></i>	<i>Gm. N<sub>2</sub>/hr.</i>
Basal	65.4	190	1.007	0.342	0.650
	Approximately 600 cc. water, no urea				
1st hour	61.5	364	1.005	0.207	0.753
2nd hour	66.2	428	1.004	0.176	0.753
3rd hour	66.0	410	1.004	0.221	0.906
4th hour	65.3	258	1.006	0.277	0.715
Total change — 2.6 Cal.					
1/24/35					
Basal	62.8	120	1.010	0.580	0.696
	30 gm. urea in approximately 500 cc. water				
1st hour	63.9	215	1.007	0.636	1.367
2nd hour	64.2	111	1.013	1.422	1.578
3rd hour	61.8	144	1.010	1.162	1.673
4th hour	62.3	230	1.005	0.613	1.410
Total change + 1.0 Cal.					
<i>Subject IV, white male</i>					
7/19/34					
Basal	62.6	85	1.007	0.409	0.348
	20 gm. urea in 400 cc. warm water				
1st hour	61.2	283	1.005	0.314	0.889
2nd hour	63.2	72	1.015	1.033	0.744
3rd hour	62.4	48	1.021	1.413	0.678
4th hour	61.8	37	1.023	1.570	0.581
Total change — 1.0 Cal.					
7/30/34					
Basal	58.6	187	1.006	0.258	0.483
	30 gm. urea in 400 cc. warm water				
1st hour	57.2	308	1.005	0.403	1.240
2nd hour	67.4	120	1.014	0.999	1.199
3rd hour	57.8	130	1.012	0.904	1.175
4th hour	63.0	330	1.005	0.294	0.970
Total change + 12.0 Cal.					
12/14/34					
Basal	60.5	119	1.008	0.168	0.200
	30 gm. urea in 400 cc. warm water				
1st hour	60.9	240	1.008	0.684	1.642
2nd hour	61.9	120	1.014	1.218	1.462
3rd hour	64.5	108	1.016	1.224	1.356
4th hour	62.8	97	1.013	1.184	1.148
Total change + 7.1 Cal.					
12/21/34					
Basal	60.5	46	1.018	0.921	0.424
	400 cc. warm water no urea				
1st hour	58.7	175	1.007	0.331	0.579
2nd hour	59.4	194	1.004	0.269	0.523
3rd hour	62.4	105	1.008	0.400	0.420
4th hour	63.2	63	1.012	0.560	0.353
Total change + 1.5 Cal.					

## RESULTS

The table is so nearly self explanatory that only a few points must be mentioned. In all of the experiments on subject I, a total increase in heat production is seen only on January 14, 1935 and that amounted to but 3.9 Cal. It will be noted that on this day no urea was ingested. This rise is in contrast to a fall of 7.7 Cal. on January 9, 1935 which was another control day. Both are well within the range of accuracy of the method. All of the experiments on subject I with the exception of that performed on January 18, 1935 show little concentration of urine as indicated by the specific gravity. This last experiment meets all the requirements of Borsook and Winegarden. There is a very great concentration of the urine. Assuming that the basal nitrogen excretion would have remained constant on this day at 0.162 gm. per hour, the excess nitrogen elimination was 2.382 gm. Calculating the expected increase of 6 to 11 Cal. per gram of nitrogen the least net increase in heat production should have been 14.3 Cal. and the maximum 26.2 Cal. This is in contrast to a net decrease of 1.0 Cal.

The only net increase in metabolism shown in the experiments on subject II was on January 23, 1935 when no urea was ingested. It would seem that the basal metabolism on that day was too low. This subject showed the ability to excrete large amounts of nitrogen and yet the metabolism failed to rise.

Subject III showed a net increase in heat production only on June 29, 1934 and this was too slight to be of any significance. There was a fair diuresis on this day in contrast to January 24, 1935 when the urine was markedly concentrated and only a slight rise occurred.

All of the experiments on subject IV showed slight net increase in heat production except that done on July 19, 1934. The greatest rise was on July 30, 1934 and a large part of this was due to the high metabolism during the second experimental hour. In all other respects this hour differed little from the third experimental hour on the same day. The more

frequent fluctuations in the case of this subject can probably be explained as being due to his lack of ability to relax quickly.

#### DISCUSSION

Our results are in accordance with those of Lusk and Grafe which are quoted above. They are in contrast to those of Lublin, to one experiment of Tangl in which urea was administered intravenously and to those of Borsook and Winegarden. Lublin administered large quantities of water to his subjects and the increases in heat production which he obtained were usually coincident with diuresis. Hence in his study the work of the kidney in the concentration of the urine could not be an important factor. It seems more probable that the explanation of his results lies in osmotic changes in the blood and tissues. The same explanation probably holds for the experiment of Tangl which is mentioned above. That osmotic changes do increase metabolism has been clearly shown by Wilhelmj, Bollman and Mann ('31). The experiments of Borsook and Winegarden are open to criticism since they used untrained subjects and a clinical apparatus not designed for extreme accuracy.

Our experiments rule out the work of the kidney in the concentration of the urine as a factor in the specific dynamic action of proteins. They do not eliminate the kidney as a possible seat for other reactions dealing with the increased heat production following ingestion of proteins or amino acids. Lusk ('31) has concluded that the cause of the specific dynamic action of proteins is probably the various intermediary reactions through which the amino acids pass during their metabolism in the body. We are now attempting to investigate whether or not some of these may occur in the kidney.

#### CONCLUSION

The work done by the kidney in the concentration of the urine during rapid excretion of urea is not a material factor in the specific dynamic action of proteins.



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# THE EFFECT OF MALE HORMONE ON THE PROTEIN AND ENERGY METABOLISM OF CASTRATE DOGS<sup>1</sup>

CHARLES D. KOCHAKIAN AND JOHN R. MURLIN

*Department of Vital Economics, University of Rochester, Rochester, New York*

FIVE FIGURES

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## INTRODUCTION

The relationship of the male gonads to metabolism has been the subject of investigation of many workers. Korenchevsky ('25) in the first of a series of articles on sex glands and metabolism, summarizes the results of castration on energy and protein metabolism obtained by various workers on both animals and humans since 1898. The results of the various investigators vary. Korenchevsky's ('25) own investigations indicated that after castration two types appear, a 'fat' and a 'thin,' in both humans and dogs. In fat castrated dogs energy metabolism and nitrogen metabolism were both decreased after castration. On the other hand in 'thin' castrated dogs this fall in the nitrogen and gaseous metabolism was usually less pronounced or absent; moreover, the gaseous metabolism was sometimes considerably increased.

Koch ('32) reviewing the results obtained by numerous workers after the injection of testis emulsions and extracts was of the opinion that the evidence for a gonadal hormonal influence on metabolism was very unconvincing.

<sup>1</sup> This investigation was aided by a grant from the Committee on Scientific Research of the American Medical Association.

Schrire and Zwarenstein ('32 a) reported that castration of adult male rabbits causes an increase of 25 to 50 per cent in creatinine output. This abnormality could be corrected by the grafting of testis and by the injection of saline suspensions or extracts of testes ('32 b).

Read ('21) found that the metabolism of Pekin eunuchs was greatly deranged like that of a cretin. Shen ('25) on the other hand was unable to find any creatine or other abnormalities in the nitrogen metabolism of eunuchs.

McCullagh, McCullagh and Hicken ('33) in a study of the clinical treatment of hypogonadism with male sex hormone, reported that after treatment there was a rise in basal metabolism and a fall in the level of blood cholesterol.

In view of this conflicting and unconvincing evidence, this study was undertaken. It has already been amply proven that the testes elaborate a hormone or hormones, presumably excreted in the urine, which affect the secondary male characteristics. It was considered advisable, therefore, to attack the problem of a gonadal hormonal relationship by studying first the effect of this male sex hormone or hormones on the metabolism of castrate dogs.

#### PREPARATION OF THE HORMONE

Since male sex hormone can be readily prepared from urine and that source of supply was conveniently available,<sup>2</sup> the starting material used in this study was urine. The method of Funk and Harrow ('30) was found to be easily adaptable and this method with two modifications was used.

The urine, which was collected in 18-liter bottles with or without preservative, was made acid by the addition of concentrated hydrochloric acid and then an additional 20 cc. of acid per liter of urine was added. Funk and Harrow ('30) found that extraction of urine so treated always resulted in the formation of an emulsion between the solvent and the urine. We were able to successfully overcome the formation

<sup>2</sup>The authors wish to express their appreciation to the medical students for their cooperation.

of this emulsion by concentrating the acidified urine to about one-eighth its volume in large evaporating dishes on an active steam bath. If, however, the urine was not acidified before concentration, or the steam bath was not very active, the emulsion would still be formed. Evidently, therefore, there is a breakdown of the emulsifying agents in the urine under the influence of acid and heat. The concentrated urine was extracted under reflux for 10 to 12 hours with chloroform, the chloroform was separated from the watery layer and the chloroform removed by distillation. The residue was heated under reflux for about 1 hour with 1 gm. of sodium hydroxide dissolved in 5 cc. water for each 3 liters of urine. The alkaline residue was then cooled and transferred to a separatory funnel with several small washings of water and ether. The diluted residue (dilution three to four times) was extracted eight to ten times with ether. If the ether was removed at this point and the residue was dissolved in olive oil, there would be considerable tarry material which would not dissolve in the olive oil. If, however, the ether extract was washed twice with dilute hydrochloric acid, the insoluble material would readily precipitate out. The ether extract was then washed twice with water, the ether removed and the final product, a reddish viscous oil, was completely soluble in olive oil, but on allowing to stand in the refrigerator for 3 to 4 days, more of the above inert material precipitated out.

The method as finally adopted has yielded quantitative results as determined by the extraction of approximately 4000 liters of urine in batches of 1 to 1000 liters.

#### ASSAY OF THE HORMONE

The hormone was dissolved in olive oil so that 1 cc. of the oil solution was equivalent to 4 liters of urine. The potency of the solution was determined qualitatively by the regeneration of the prostate and seminal vesicles of castrate rats and quantitatively by its effect on the combs of White Leghorn capons, according to the method of McCullagh, McCullagh and Hicken ('33). The hormone solution, 0.05 cc., was injected

into the pectoral muscles for 2 successive days and a maximum growth of length plus height ( $L + H$ ) was obtained on the fourth day. The results obtained are listed in table 1 and are the same for the different preparations, i.e., 25 to 34 B.U. per cubic centimeter according to the McCullagh technic.

#### DESCRIPTION

Three dogs were used. Dog 1 was castrated in November, 1931 and has always been thin and very active. Dog 2 was castrated in November, 1933 and soon became fat and very inactive, the direct opposite of dog 1. Dog 3 was castrated<sup>3</sup> in November, 1934. At the time of castration it was ap-

TABLE 1  
*Assay of the hormone preparations (0.05 cc. injected per day)*

PREPARATION NUMBER	DATE	INCREASE OF LENGTH + HEIGHT	AVERAGE INCREASE
II	2/4/35	+ 6, + 4, + 5	+ 5
III	2/4/35	+ 5, + 4, + 6	+ 5
X	12/11/34	+ 8, + 4, + 7	+ 6
	1/7/35	+ 6, + 6	+ 6
	3/27/35	+ 4, + 4	+ 4
	4/17/35	+ 6, + 4	+ 5
67	4/17/35	+ 5, + 5, + 5, + 5 (+ 11)	+ 5

proximately 10 months old and had been in confinement for 4 months. Castration to date has not shown any significant effect on its basal metabolism, as calculated by the Cowgill-Drabkin method ('27) for Cal./sq.m./hr. This dog has not shown any tendency to become a fat castrate.

*Diets.* The dogs were maintained on diets which proved adequate in all respects and at no time did the dogs show any impairment of appetite or other dietary deficiencies.

Dogs 1 and 2 were placed on the diet shown in table 2.

The nitrogen content of the beef heart varied but analyses were made of each beef heart at the time of feeding. The cracker meal was kept in a tightly sealed glass container. All

<sup>3</sup> The authors wish to express their appreciation to Mr. Teufel and Mr. Disbro, medical students, for their assistance in training and castrating this dog.

analyses were made on aliquots by the Kjeldahl-Gunning method.

The diet was prepared by melting the lard to which was added the beef heart, previously cut into small squares, and the other constituents plus about 700 cc. of water.

TABLE 2  
*Beef heart diet*

	DOG 1, WEIGHT 12 KG.			DOG 2, WEIGHT 22 KG.		
	Grams	Grams nitrogen	Calories	Grams	Grams nitrogen	Calories
Beef heart	150	4.50	372	200	6.00	500
Cracker meal	60	1.16	233	85	1.67	332
Lard	20	....	180	24	....	180
Cod liver oil	5	....	45	5	....	45
Bone ash	5	....	....	10	....	....
Wesson's salt mixture	3	....	....	3	....	....
Totals		5.66	830		7.67	1057

TABLE 3  
*Cowgill's diet*

	DOG 3, WEIGHT 20.9 KG.		
	Grams	Grams nitrogen	Calories
Casein	109.3	13.79	405.7
Sucrose	102.3		409.2
Bone ash	4.8		
Wesson's salt mixture	2.4		
Yeast powder <sup>1</sup>	1.2		4.0
Lard	50.0		450.0
Cod liver oil	5.0		45.0
Totals		13.79	1313.9

<sup>1</sup> Standard brand unirradiated.

Dog 3 was placed on the diet prescribed by Cowgill ('23).

The first five constituents of the casein diet were prepared in a large batch and placed in a tight tin container. Aliquots of this mixture were analyzed for nitrogen. The diet was prepared by mixing the dry constituents and cod liver oil with the melted lard.

## PROCEDURE

The dogs were confined in metabolism cages and fed the prescribed diet at approximately 4 P.M. every day. The dogs were catheterized just before feeding daily or at the end of each period (5 days each) depending on the type of experiment. The urine was collected under toluene daily and saved in the refrigerator until the end of the period when analyses were made. Total nitrogen of the urine was determined by the Kjeldahl-Gunning method, ammonia and urea by the Van Slyke-Cullen ('16) aeration method, creatine and creatinine by the Folin ('14) microchemical method. The rest nitrogen was determined by difference.

The fecal periods were terminated with the use of carmine as a marker. The feces were collected daily and saved in the refrigerator in acidified alcohol. At the end of the period the feces were dried on a steam bath, powdered and aliquots analyzed for nitrogen.

The energy metabolism of dogs 1 and 3 was determined by means of a Benedict closed circuit apparatus, attached to an all metal chamber, which was enclosed by a water jacket. Frequent alcohol checks were made to be certain that the apparatus was air-tight and working properly.

The Tissot-Haldane method with face mask was used for dog 2.

The three dogs were thoroughly trained to their respective regimes. Activity records of dogs 1 and 3 were obtained by means of a kymograph. Dog 2 was kept under observation and respiration rates recorded. Only those experiments were used in which the dogs were quiet and in a basal condition. Dogs were fed regularly at 4 P.M. and all energy metabolism experiments were begun at least 17 hours later. Temperature of the chamber was always between 22° and 27°C. and, except in the case of experiment no. 1 on dog 1, as mentioned on page 447, the animals were kept in the laboratory throughout the experimental period so that no temperature readjustment was in question (aside from the instance given).

## RESULTS

*Successive injections*

The experiments on protein and energy metabolism were carried out simultaneously. At first, however, we were only concerned with the energy metabolism. Therefore, preliminary experiments were made on the effect of large single injections on energy metabolism alone. Since these experiments, which will be discussed later, yielded negative results, continuous injections for a period of time were carried out. Four such experiments were carried out with dog 1 and three with dog 2. A summary of the nitrogen balances of these experiments are presented in table 4.

TABLE 4  
*Summary of nitrogen balances, grams per period (5 days)*

Bird units (B.U.) male hormone per day	DOG NO. 1				DOG NO. 2		
	13-17	25-34	38-51	25-34	25-34	38-51	13-17
Experiment no.	1	2	3	4	1	2	3
Periods before injection							
1	-0.10	+0.30	+0.05	+0.65	+4.75	+0.45	
2			+0.80	+1.30		+1.10	+0.45
Injection periods							
1	+1.45	+2.65	+2.55	+2.40	+4.50	+2.90	+1.70
2	+3.05	+3.45	+2.35	+3.10	+6.35	+6.45	+0.85
3	+2.85	+5.05	+0.50	+4.25	+6.45	+6.55	+1.65
4			+2.45		+6.60	+6.55	+2.60 <sup>a</sup>
5			+3.65				
Periods after injection							
1	+3.15	+3.00	+1.30	+4.20 <sup>1</sup>	+4.05	+3.90	+0.55 <sup>a</sup>
2	0.00	+0.75	-1.00	+1.90	+1.00	-2.75	-0.45
3		+0.55 <sup>1</sup>	+0.65	+2.10	-1.75 <sup>4</sup>	-1.50	-0.35
4			+1.30	+2.50	-1.30	-1.45	0.00
5				+0.70	+0.40 <sup>3</sup>	-0.65	
6				+2.60	-1.55	-0.30	
7					0.00	-1.20	
8					-0.50	-1.05	
9					+0.45	+0.45	
10					+1.10		

<sup>1</sup> Four-day period, but calculated for 5 days.

<sup>2</sup> Six-day period, but calculated for 5 days.

<sup>3</sup> Seven-day period, but calculated for 5 days.

<sup>4</sup> Three days lost between this and the preceding period.



In the seven experiments recorded, a single preparation, X, which was assayed repeatedly (table 1, p. 440) during the course of the experiments was used. No toxic effects were noted and the hormone solution was readily absorbed. In every experiment except the last, the hormone was injected subcutaneously in half doses every morning and evening. In the last experiment the hormone was injected in a single dose every morning.

The results for dog 1 indicate that there is an increased nitrogen retention with increased dose up to a certain point (experiment 2), beyond which (experiment 3) there is no greater response and possibly a decrease. Experiment 4 was run immediately after experiment 3 to see if experiment 2 could be duplicated. As figures show, there is fairly good duplication.

Experiments 1 and 2 of dog 2 yielded remarkably constant and identical results. Undoubtedly, this is due to obtaining a maximum effect with the first dose, beyond which no greater effect resulted. The third experiment, therefore, was designed to determine whether a smaller dose would produce the same effect. As may be seen, table 4, decreasing the dose to half that of experiment 1, produced only about 30 per cent the effect. The high basal value obtained in experiment 1 was due to a personal fault in that sufficient time was not given the dog to come into nitrogen equilibrium. It does not, however, detract from the significance of the results, for comparatively it affected only the first injection period.

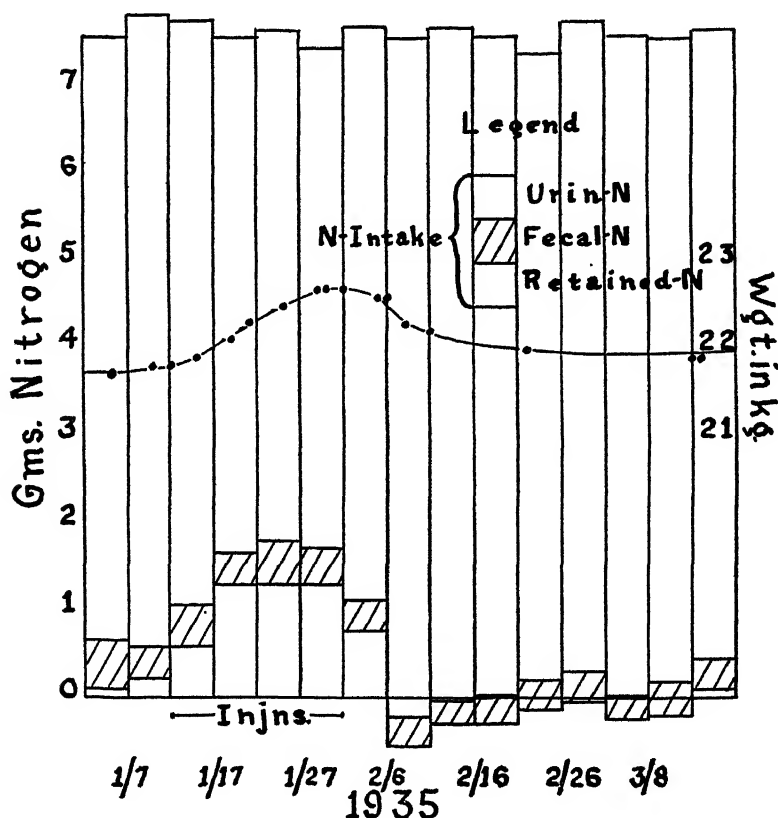
In every case dog 2 went into a sustained minus balance shortly after cessation of injections. This minus balance attained a maximum immediately and gradually returned to basal. The excess nitrogen output, however, never attained the proportions of the amount retained. In experiment 3 the amount and duration of the minus balance is correspondingly shorter.

The three experiments were run successively.

The graphical presentation of experiment 2 of dog 2, (graph 1) illustrates clearly the effect of male sex hormone on the

nitrogen balance and also on the dog's weight. The values are represented so that the nitrogen intake is divided into urinary nitrogen, fecal nitrogen and nitrogen retained. The latter, of course, only when the dog is in positive nitrogen

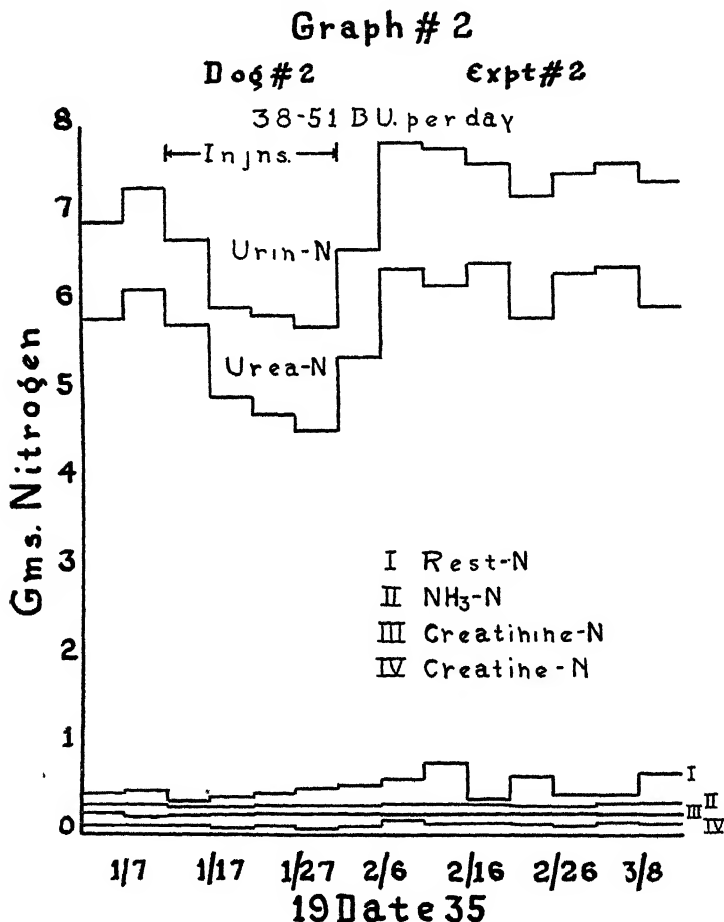
Graph #1



balance. The changes in the nitrogen balance are borne by the urinary nitrogen and never by the fecal nitrogen. The weight of the dog gradually increases and then gradually returns to basal after cessation of injections.

In order to determine what constituents of the urinary nitrogen were affected, partitions of the urinary nitrogen

were made. Graph 2 shows typical results. The significant fact to be noted is that the urea nitrogen parallels the changes in urinary nitrogen. The ammonia and creatinine remain constant. The rest nitrogen shows a variable increase and



creatine nitrogen always increases slightly after cessation of injections.

#### ENERGY METABOLISM

Although there is a similar effect on the protein metabolism of both dogs, the effect on the total energy output is somewhat different. Tables 5 and 6 summarize the results for

dogs 1 and 2, respectively. The values in table 5, dog 1, are for total R.Q.'s, the values in table 6, dog 2, are calculated for non-protein R.Q.'s according to Lusk ('24, '28). The calories per square meter per hour are calculated according to the surface area formula for dogs proposed by Cowgill and Drabkin ('27).

TABLE 5  
*Summary, energy metabolism in dog 1*

EXPERIMENT NO.	DATE	NUMBER DETERMINATIONS	TOTAL R.Q.	CALS./SQ.M./HR.	WEIGHT AT END OF PERIOD	REMARKS
1	4/15-4/18/34	3	$0.74 \pm 0.01$	$30.9 \pm 0.4$	13.4	Basal
	5/5-5/16	11	$0.78 \pm 0.02$	$29.4 \pm 0.5$	13.5	13-17 B.U. per day ( $\frac{1}{2}$ cc. X) 5/3-5/18
	5/20-5/25	6	$0.79 \pm 0.02$	$28.3 \pm 1.6$	13.4	Basal
2	6/26-6/27	3	$0.77 \pm 0.02$	$28.9 \pm 0.7$	12.8	Basal
	6/28-7/12	5	$0.75 \pm 0.01$	$29.1 \pm 1.0$	13.1	25-34 B.U. per day (1 cc. X) 6/28-7/12
	7/14-7/27	5	$0.75 \pm 0.02$	$28.6 \pm 1.1$	12.9	Basal
3	12/13-12/20	6	$0.77 \pm 0.02$	$29.7 \pm 0.9$	11.9	Basal
	12/20-1/14-35	8	$0.75 \pm 0.02$	$29.4 \pm 1.0$	12.2	38-51 B.U. per day ( $1\frac{1}{2}$ cc. X) 12/20-1/14
	1/15-2/1	7	$0.76 \pm 0.01$	$28.6 \pm 1.2$	11.9	Basal
4	2/5-2/15	5	$0.75 \pm 0.01$	$29.4 \pm 0.9$	12.1	25-34 B.U. per day (1 cc. X) 2/3-2/18
	2/20-3/20	5	$0.76 \pm 0.01$	$29.2 \pm 0.6$	12.0	Basal

Dog 1 shows a remarkable constancy in every experiment with the exception of no. 1, which shows a gradual decrease. This, however, is probably not due to the effects of the hormone but to the fact that the dog was acclimating itself to the temperature of the laboratory after its stay in the animal house, which was much cooler.

Dog 2, table 6, on the other hand, shows definite increase in total energy output in two of the three experiments after administration of the hormone. Experiment 3 shows a smaller and rather doubtful increase. It must be emphasized that the increase is never very great. The increase in energy metabolism does not begin immediately, but only after several days;

TABLE 6  
*Summary, energy metabolism dog 2*

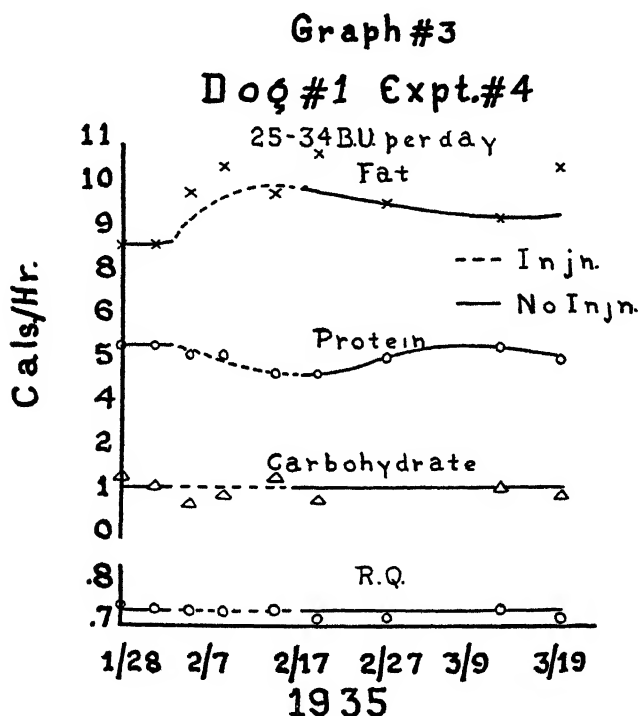
EXPERIMENT NO.	DATE	NUMBER DETERMINATIONS	NON PROTEIN R.Q.	CALS./SQ.M./HR.	WEIGHT END OF PERIOD	REMARKS
					<i>kg.</i>	
1	10/31/34					25-34 B.U. per day (1 cc.X) 10/31-
	11/14-11/20	7	$0.78 \pm 0.01$	$26.2 \pm 0.7$	22.2	11/20/34
	11/21-11/26	4	$0.79 \pm 0.02$	$25.2 \pm 0.7$	22.4	Aft. injection
	12/1-1/10/35	11	$0.78 \pm 0.03$	$24.1 \pm 0.7$	21.8	Basal
	1/12-1/15	2	$0.74 \pm 0.03$	$24.5 \pm 0.1$	21.8	38-51 B.U. per day (1½ cc. X) 1/12-
2	1/19-2/1	17	$0.73 \pm 0.03$	$27.1 \pm 0.5$	22.7	2/1
	2/6-2/16	5	$0.77 \pm 0.04$	$26.1 \pm 0.2$	22.2	Aft. injection
	3/4-3/14	7	$0.82 \pm 0.02$	$24.6 \pm 0.5$	21.9	Basal
	3/21-4/6	13	$0.82 \pm 0.02$	$25.4 \pm 0.5$	22.3	13-17 B.U. per day (1 cc. X) 3/18-
						4/16
3	4/8-4/15	4	$0.83 \pm 0.02$	$25.4 \pm 0.0$	22.3	Aft. injection
	4/18-4/24	6	$0.85 \pm 0.02$	$23.0 \pm 0.5$	22.3	Basal

later it attains a maximum and continues at that rate for a time after cessation of injections.

In order to determine whether the daily injections had any transient effect, determinations were made on both dogs before the morning injection and at various time intervals after the injections. No effects were detected. Therefore, the increase noted for dog 2 is due to the stimulation of the repeated injections.

In experiment 1 no determinations were made before or during the first part of the injection period. The subsequent results and the results obtained in experiments 2 and 3 substantiate the results obtained in experiment 1.

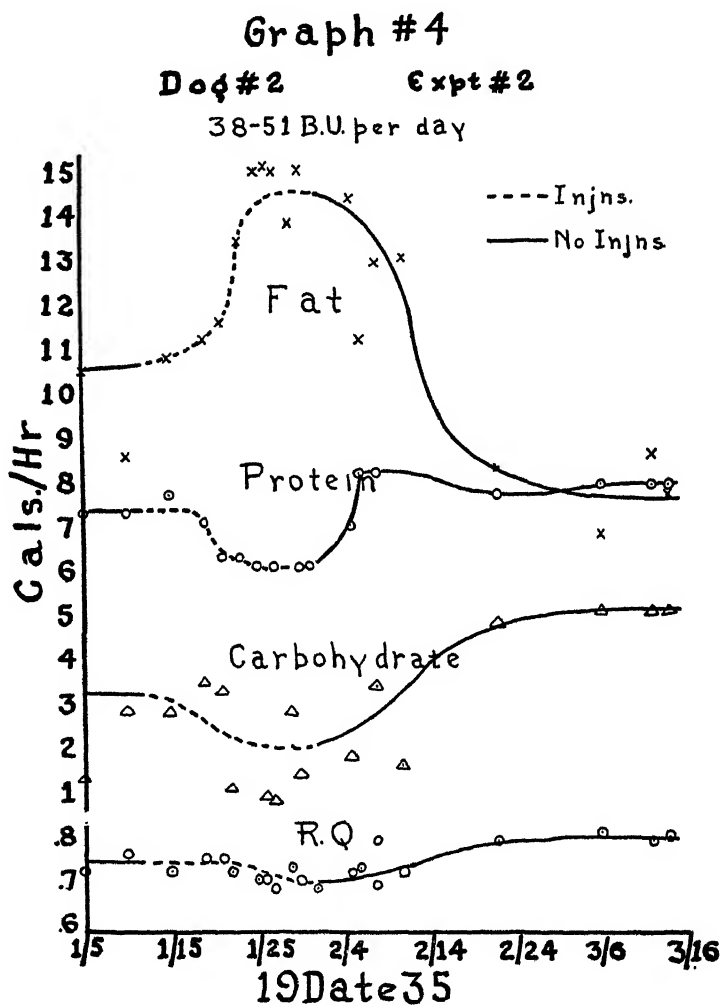
Analysis of the non-protein calories by means of the table given by Lusk ('24, '28) shows that in the case of dog 1 the fat metabolism increases to compensate for the decreased



protein metabolism (graph no. 3). The carbohydrate metabolism which is not very great to begin with, is not materially affected. Dog 2 (graph no. 4) on the other hand, shows a sparing effect in the carbohydrate metabolism as well as the protein metabolism and an increase in fat metabolism to compensate for these sparing effects and also to bear the increased total energy output.

*Single large injections*

In view of the marked effect obtained on the protein metabolism of both dogs after the continuous injection of male



sex hormone, it seemed advisable to reinvestigate the effect of single injections on the energy metabolism and at the same time observe the protein metabolism.

In the preliminary experiments in which only the energy metabolism was studied, the results in table 7 are typical.

As may be seen, there is no effect as much as 6 hours after the injection. Subsequent experiments in which observations were made as much as 11 hours after the injection and on subsequent days, showed similar results. The doses used were 33 cc. (825 to 1120 B.U.) preparation III, dog 1; 8 cc. (200 to 272 B.U.) preparation II, and 9.1 cc. (208 to 310 B.U.) preparation 67, dog 2; and 5 cc. (125 to 170 B.U.) and 9.4 cc. (235 to 319 B.U.) preparation 67, dog 3.

A study of the protein metabolism in one of these experiments on dog 2, injection of 9.1 cc. (208 to 310 B.U.) preparation 67, and one on dog 3, injection of 9.4 cc. (235 to 319

TABLE 7  
*Energy metabolism dog 1*

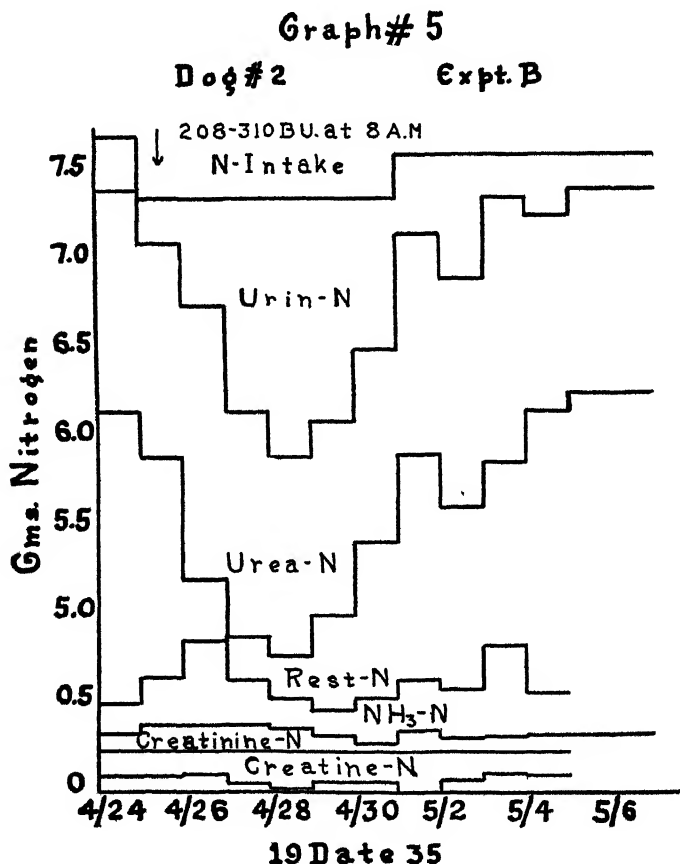
TIME	TOTAL R.Q. <sup>1</sup>	CALS./SQ.M./HR.
2/22/34 weight 14.2 kg.		
10.05 A.M.	0.73	35.6
11.10	10 cc. preparation II (50 l. urine)	
12.01-12.58 P.M.	0.75	34.8
12.58- 2.00	0.73	37.0
2.00- 3.04	0.74	35.3
3.04- 4.03	0.74	35.0
4.03- 5.00	0.77	35.3
2/27/34 basal		
10.48-11.43	0.74	35.2

<sup>1</sup> EtOH check 3/16/34—0.665, 0.664.

B.U.) preparation 67, showed very striking results, as illustrated for dog 2 in graph 5. There is a marked change in urinary nitrogen. On the day of injection there is no effect. The apparent drop on this day in urinary and urea nitrogen is proportional and consequently due to the decreased nitrogen intake, as is evident from the graph. The day after injection, however, shows a decrease which becomes successively greater and reaches a maximum on the third day and then returns in a similar manner to normal. Partition of the urinary nitrogen shows the urea nitrogen paralleling the changes in the total urinary nitrogen, while the creatinine nitrogen remains abso-



lutely constant, and creatine nitrogen, in contrast to its behavior during small daily injections, changes in parallel with the urinary and urea nitrogen, except for 1 day, May 1 to May 2. Ammonia nitrogen shows no striking effect and the rest nitrogen shows a variable effect. As with repeated injections the nitrogen retained is obviously only a part of



the amino acid nitrogen which, without the influence of the hormone, would have been deaminated and converted to urea. Rest nitrogen is made up in part also of amino acid nitrogen. The stimulus to growth of the accessory structures of the sexual apparatus has laid claim to the amino acids before they could be deaminated or excreted.

In view of this discrepancy in the action of creatine after continuous daily injections and a single large injection, and the fact that these castrate dogs were excreting creatine, an abnormality possessed by castrates, according to Read ('21) but disputed by Shen ('25), it seemed desirable to eliminate the possibility that the excreted creatine might be of exogenous origin, since beef heart contains considerable creatine. Now dog 3, prior to castration had been placed on a diet similar in every respect to that of dog 1 and dog 2 (table 2) and analysis of its urine at the time indicated an abnormal creatine to creatinine ratio of 1.29. Therefore, dog 3 was placed on a creatine free diet, Cowgill's diet for dogs ('23)

TABLE 8  
*Dog 3. Creatine and creatinine excretion (Cowgill's diet<sup>2</sup>)*

Date (1935)	5/7	5/8	5/9 <sup>1</sup>	5/10	5/11	5/12
Gm. creatine N	0.016	0.010	0.014	0.026	0.007	0.026
Gm. creatinine N	0.204	0.198	0.191	0.197	0.198	0.198

Date (1935)	5/13	5/14	5/15	5/16	5/17	
Gm. creatine N	0.003	0.007	0.008	0.002	0.006	
Gm. creatinine N	0.197	0.194	0.200	0.195	0.205	

<sup>1</sup> Injected 9.4 cc. of preparation 67 at 8.00 A.M.

<sup>2</sup> Creatine/creatinine = 1.29 on beef heart diet.

(table 3), with casein the sole source of protein. A study of the nitrogen metabolism before and after the injection of 9.4 cc. (235 to 319 B.U.) of preparation 67, yielded results the same as for dog 2 with the one exception that only traces of creatine could be detected both before and after the injection (table 8).

#### DISCUSSION

Although in the interest of brevity the complete data have not been presented, the summaries and typical experiments shown adequately explain the results of this investigation.

It is to be noted that the nitrogen retained is never completely excreted. In fact dog 1, the thin castrate, during only

one period, 5 days, in the four experiments, goes into a negative balance. Dog 2, on the other hand, always goes into a negative balance after cessation of injections and gradually returns to equilibrium. Undoubtedly the nitrogen is retained as circulating (or reserve) protein, but it seems difficult to understand why dog 2 should excrete part of the retained nitrogen and dog 1 none, unless we assume that in the latter at least part of the retained nitrogen has been converted to permanent structures.

The difference between dogs 1 and 2 appears to be similar in some respects to that between a pregnant dog in the late weeks of gestation and a non-pregnant dog. Fed high protein both dogs retain nitrogen; when returned to low protein the non-pregnant dog excretes more than the pregnant. A closely parallel situation is found also in menstruation followed by pregnancy (Murlin, '10) where the retention of nitrogen under the influence of oestrin is followed by a minus balance in the early weeks of gestation. Oestrin is no longer effective to produce retention. In the present experiments male hormone causes retention in both dogs, but dog 2 does not retain so tenaciously as dog 1. Dog 2, being fat, suggests a relative pituitary deficiency. Injection of the globulin fraction of the Bugbee extract of anterior pituitary at the end of male hormone injections in the case of dog 2, certainly would have converted the minus balance periods into positive (Gaebler, '33). Gaebler, in fact, finds that the effect of the growth hormone in dogs varies much as the effect of the male hormone does in this study. Sometimes the retention is permanent, at other times temporary, suggesting that some additional factor is necessary to cause conversion of reserve protein into cell structures.

The significant effect on the urinary constituents is the paralleling of the changes in the urinary nitrogen by the urea nitrogen, which was to be expected in decreased catabolism of exogenous protein. The outer urinary constituents do not show any striking differences except possibly creatine.

At first it seemed that the finding of appreciable amounts of creatine in the urine of these castrate dogs confirmed the findings of Read ('21). An investigation, however, as to the source of the creatine excreted indicated that it came from the diet. When the diet did not contain creatine the urine also was free of creatine. Our results on the creatine metabolism of castrates confirm those of Shen ('25) rather than Read ('21).

The creatinine output at no time was affected and in fact remained remarkably constant which cannot be reconciled with the findings of Schrire and Zwarenstein ('32 a and b).

The ammonia nitrogen was always normal with a slight variance which is not significant.

Comparison of the results obtained on the nitrogen retention after the administration of repeated doses and single large doses, indicates clearly that the former is the more efficient method. The administration of a dose of 9.1 cc. of preparation 67 produced in dog 2 a maximum retention of 15.5 per cent, while the injection of  $\frac{1}{2}$  cc. of preparation X, twice per day, produced a maximum nitrogen retention of 17 per cent in less than 5 days. Therefore the same effect was produced with less than half as much hormone. There is also a point beyond which increasing the amount of the hormone does not increase the amount of nitrogen retained.

The different results obtained in the heat production of the two dogs can be explained at present only by the difference in their tendencies to become fat. Dog 2, weighing around 22 kg., has a basal heat production of 24 Cal./sq.m./hr., while dog 1, weighing around 12 kg. has a basal of 29 Cal./sq.m./hr., i.e., 20 per cent greater. Injection of male hormone compensates for castration in the fat dog more than in the thin. It must be borne in mind, however, that the increase in heat production produced in dog 2, and this only after several days of repeated injections, is only about 10 per cent. It is significant, therefore, that while the male sex hormone produces a change in the protein and fat metabolism, and a decrease in carbohydrate metabolism in the fat castrate dog, it is not a calorogenic agent of importance.

The difference noted in the energy metabolism of the two dogs and the protein metabolism—the fact that dog 1 does not excrete the nitrogen retained while dog 2 excretes part of its retained nitrogen—suggests the participation of some other endocrine organ.

Furthermore, the striking similarity of our results on nitrogen metabolism, but not on energy metabolism, after the injection of male sex hormone, with those of Teel and Cushing ('30) and Gaebler ('33) after the injection of anterior pituitary growth hormone, suggests a metabolic interrelationship of the gonads and the anterior pituitary.

Both dogs always increased in weight—about  $\frac{1}{2}$  kg. dog 1; about 1 kg. dog 2—with a gradual return to preinjection level. The increased body protein is not sufficient to explain these increases. It is possibly due to a gradual hydration and a subsequent dehydration, for no marked thirst or diuresis was noted.

The male sex hormone prepared from urine has not as yet been proven to be chemically or physiologically identical with that produced by the extraction of testicular tissue. Prevailing evidence indicates that not only are the substances produced from the two sources different chemically, but also physiologically. Butenandt ('31, '34) has prepared male sex hormone from urine in pure crystalline form and designated it androsterone. Ruzicka ('34) has prepared an identical product synthetically from cholesterol. Ogata and Hirano ('34) have prepared from boars' testes pure crystalline material which is chemically different from androsterone. Koch and Gallagher ('34) report that while the product from urine is stable toward heating with alkali, the product from beef testes is destroyed. Dingemanse, Freud and Laqueur ('35) reported definitely different effects of the two products on the amount of regeneration of prostate and seminal vesicles of castrate rats. Kozelka and Gallagher ('34) and Willier, Gallagher and Koch ('35) report that extracts of bull testes injected into chick embryos produced no change, while urinary extracts produced normal ovarian tissue in the cortical portion of the left gonad.

Early in our investigations it occurred to us that probably the hormone prepared from the testicular tissue was different and would produce a calorigenic effect. Preliminary experiments suggest this possibility.

#### ADDENDUM

David et al. ('35) in a preliminary note, report the isolation of pure crystalline hormone from testes (testosterone) which is six times as potent as pure crystalline material from urine (androsterone) and possesses different chemical as well as physiological properties.

#### CONCLUSIONS

Male hormone as prepared from urine produces the following definite results in adult male castrate dogs.

A marked drop in urinary nitrogen with a subsequent increase which are both due to changes in urea nitrogen. Other constituents show no significant changes. Creatine is not excreted by castrate dogs if on a creatine-free diet.

The decrease of urinary nitrogen does not begin until the first day after the injection. After reaching a maximum, further injections only maintain the decreased protein catabolism at this level.

It is more efficient to use small repeated doses than single large doses.

The heat production of thin castrate dogs is not affected while that of a fat castrate dog is raised slightly, 10 per cent, after repeated injections.

The fat metabolism is increased, the protein metabolism decreased and the carbohydrate metabolism is not affected in the thin dog, but is decreased in the fat dog.

The weights of the dogs show a significant but not large increase during injection, and a prompt return to the pre-injection level thereafter.

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# THE UTILIZATION OF ENERGY PRODUCING NUTRI- MENT AND PROTEIN AS AFFECTED BY INDIVIDUAL NUTRIENT DEFICIENCIES

## III. THE EFFECTS OF THE PLANE OF PROTEIN INTAKE <sup>1</sup>

E. B. FORBES, R. W. SWIFT, ALEX BLACK AND O. J. KAHLENBERG  
*Institute of Animal Nutrition, Pennsylvania State College*

### SEVEN FIGURES

(Received for publication August 2, 1935)

Two similar experiments were conducted on the plane of protein intake as affecting the utilization of food energy and protein by albino rats, this study being one of a series performed by the same original routine, especially from the point of view of an interest in measures of the nutritive values of foods.

Other studies on related subjects by the same routine have been published by Swift, Kahlenberg, Voris and Forbes ('34), McClure, Voris and Forbes ('34), and Braman, Black, Kahlenberg, Voris, Swift and Forbes ('35).

The first of the two experiments to be discussed was conducted in 1933-1934. The results appeared to be valid, but were in important details unexpected. In order to confirm or to disprove these findings, therefore, and to throw light upon the comparatively new experimental procedure employed, this first experiment was repeated, as nearly as practicable in the same way, in the years 1934-1935.

In each of these experiments a study was made, with forty-eight albino rats as subjects, of planes of protein intake varying between moderate deficiency and approximate optimum.

<sup>1</sup> Authorized for publication on July 31, 1935, as paper no. 663 in the journal series of the Pennsylvania Agricultural Experiment Station.

Four diets were compounded to contain 10, 15, 20 and 25 per cent of protein, respectively, to have exactly the same gross energy value, and to supply all known mineral and vitamin requirements. These four diets were fed one to each rat of groups of four individuals, each such group being of one sex and of the same litter, and of approximately the same initial live weight. The rats were fed in accord with the principle of paired feeding (in this case quadruplets instead of pairs); each rat of a group of four receiving the same energy but different protein intake; there being, in each experiment, twelve individuals on each of the four treatments.

The diets were composed as follows:

First experiment; constant constituents: butter fat 10 per cent, brewer's yeast 5 per cent, irradiated yeast 1 per cent, Cellu flour 4 per cent, Osborne and Mendel salt mixture 4 per cent and sodium chloride 1 per cent; and varied constituents, dextrin 51.156 to 62.325 per cent, casein 7.573 to 23.844 per cent and Crisco 0 to 5.102 per cent.

Second experiment; constant constituents: butter fat 10 per cent, brewer's yeast 5.25 per cent, irradiated yeast 0.75 per cent, Cellu flour 4.0 per cent, Osborne and Mendel salt mixture 4.0 per cent and sodium chloride 1.0 per cent; and of the varied constituents the percentages were of dextrin 51.156 to 62.325 per cent, casein 7.573 to 23.844 per cent, and Crisco 0 to 5.102 per cent.

The experimental feeding began as soon as the rats were weaned, and continued for 10 weeks.

The quantity of food given each day to each unit of four rats was determined by the quantity consumed by the individual, among the four, which ate the least. In the first experiment the total numbers of refusals of the 10, 15, 20 and 25 per cent protein rations were 213, 52, 10 and 56, respectively; and in the second experiment the numbers of refusals were 154, 97, 76 and 42, respectively. In both experiments, therefore, the 10 per cent protein diet determined the food intake for all groups.

Urine and feces were collected separately and continuously, during the 10-week period of experimentation, and were subjected to chemical analysis.

At the end of the experiments all rats were killed and analyzed, and gains of constituents were determined by comparison of data from these rats which were fed and killed with data from a control group of twelve rats killed at the beginning of the experiments.

The experiments progressed from beginning to end with unusual regularity and consistency.

TABLE 1  
*Average gain in body weight<sup>1</sup> related to dry matter of feed*

PLANE OF PROTEIN INTAKE	GAIN IN BODY WEIGHT	FOOD EATEN (DRY MATTER)	DRY MATTER OF FOOD PER GRAM BODY GAIN
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Experiment no. 1			
10% protein	78.93	437.6	5.5 ± 0.12
15% protein	107.04	437.9	4.1 ± 0.11
20% protein	119.13	438.3	3.7 ± 0.09
25% protein	119.41	438.5	3.7 ± 0.09
Experiment no. 2			
10% protein	66.04	419.7	6.4 ± 0.14
15% protein	92.85	420.9	4.5 ± 0.10
20% protein	99.55	420.4	4.2 ± 0.11
25% protein	100.35	419.2	4.2 ± 0.10

<sup>1</sup> Contents of alimentary tract removed.

NOTE: Each datum is an average value representing twelve animals on a continuous metabolism investigation during 10 weeks.

In the course of the second experiment there was observed, in three rats among the forty-eight, slight evidence of a dietary deficiency which, however, was not sufficiently marked appreciably to affect the appetite or the growth of the rats, and was not positively diagnosed.

Rats nos. 1 and 25, on the 10 per cent protein diet, and no. 18 on the 15 per cent protein diet, exhibited loss of hair, in differing degrees, from the ventral side of the body.

On this account, and with the thought that the symptom noted might possibly be due to a fatty acid deficiency, each

rat of the forty-eight was given 3 drops of cod liver oil per day from the time this disorder was observed until the end of the experiment.

This treatment led to marked improvement of the affected rats, with respect to this condition, and the cod liver oil was accounted for as a part of the energy intake.

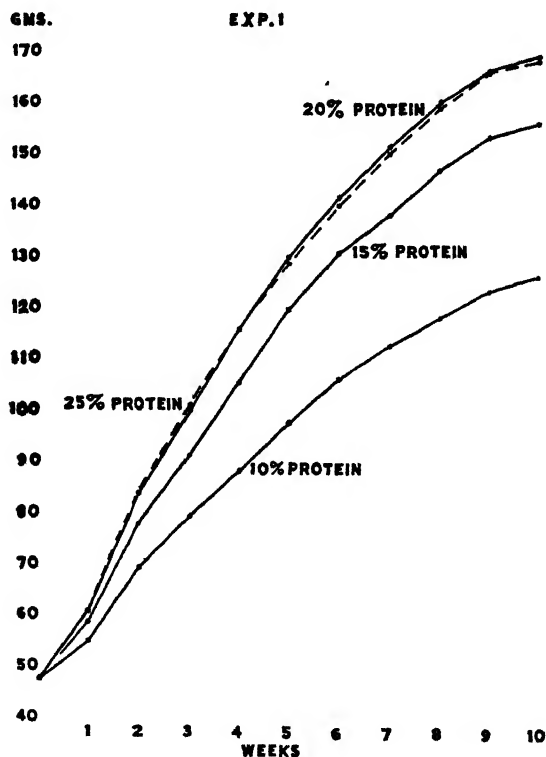


Fig. 1 Experiment 1. Average growth of albino rats during 10 weeks with the same energy but different protein intake.

Referring to table 1, and figures 1 and 2, it is clear that, with constant energy intake, the increase in percentage of protein led to greater increase in body weight, the increment in percentage of protein from 10 to 15 leading to greater increase in rate of body gain than the increment from 15 to 20 per cent, while the effects of the increment from 20 to 25

per cent of protein were so small as not to be statistically significant.

Since the energy intake remained constant the observed increase in rate of gain in body weight necessarily implied

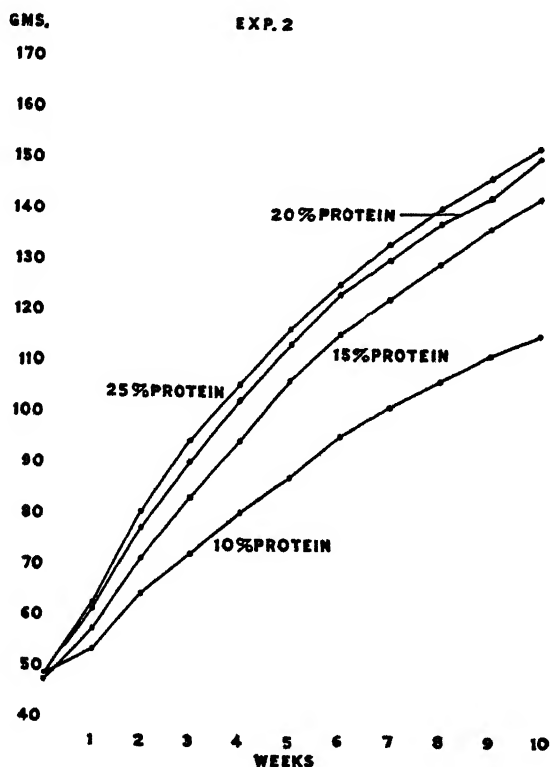


Fig. 2 Experiment 2. Average growth of albino rats during 10 weeks with the same energy but different protein intake.

a decrease in dry matter of food utilized per unit of body gain.

The rising level of protein intake from 10 to 25 per cent, therefore, brought about a progressive improvement of nutritive balance, at a diminishing rate of such improvement in relation to the increase in protein of the food.

The odds expressing the degree of significance of the gains in body weight, computed by the modification by Love ('24) of the method of 'Student' ('08), were as follows:

	<i>Experiment no.</i>	
	<i>I</i>	<i>II</i>
Gain in body weight from 15 per cent protein greater than from 10 per cent protein	10,000:1	10,000:1
Gain in body weight from 20 per cent protein greater than from 15 per cent protein	66:1	207:1
Gain in body weight from 25 per cent protein greater than from 15 per cent protein	400:1	490:1
Gain in body weight from 25 per cent protein greater than from 20 per cent protein	1:1	2:1

The gains of energy in the body growth, and the relations of the energy of the body gain to the energy and to the metabolizable energy of the food are indicated in table 2.

TABLE 2

*Average energy of body gain related to energy and to metabolizable energy of food*

PLANE OF PROTEIN INTAKE	FEED ENERGY	BODY GAIN		METABOLIZABLE ENERGY	BODY GAIN AS PERCENTAGE OF METABOLIZABLE ENERGY
			Per cent of feed energy		
	<i>Cal.</i>	<i>Cal.</i>	<i>per cent</i>	<i>Cal.</i>	<i>per cent</i>
Experiment no. 1					
10% protein	2163.6	224.2	10.4 ± 0.32	1932.7	11.6 ± 0.35
15% protein	2163.6	262.7	12.1 ± 0.50	1930.9	13.5 ± 0.53
20% protein	2163.6	295.9	13.7 ± 0.47	1938.6	15.3 ± 0.52
25% protein	2163.6	301.6	13.9 ± 0.43	1927.8	15.6 ± 0.48
Experiment no. 2					
10% protein	2075.9	226.4	10.9 ± 0.36	1858.3	12.2 ± 0.41
15% protein	2075.9	259.4	12.4 ± 0.42	1859.9	13.8 ± 0.47
20% protein	2075.9	267.0	12.9 ± 0.35	1857.4	14.4 ± 0.40
25% protein	2075.9	270.2	12.9 ± 0.42	1847.9	14.5 ± 0.47

NOTE: Each datum is an average value representing twelve animals on a continuous metabolism investigation during 10 weeks.

These data, and figures 3 and 4, show that with the same food energy intake the increase in the protein of the diets from 10 to 15 per cent, and from 15 to 20 per cent, brought about increased storage of food energy (figs. 3 and 4), and increased percentage of food energy and of metabolizable energy, as body gain (table 2); but that the increase in protein

of the diet from 20 to 25 per cent produced very little added energy retention.

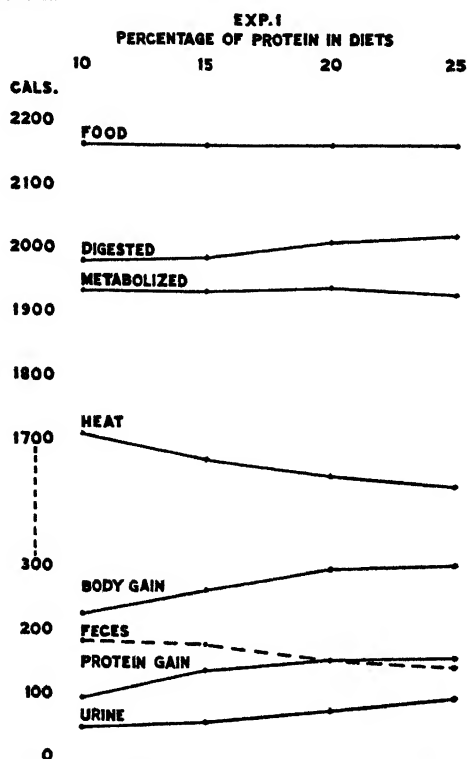


Fig. 3 Experiment 1. Average utilization of food energy during 10 weeks as affected by differences in the plane of protein intake.

The odds relating to energy gain as affected by the percentage of protein in the diets were as follows:

	Experiment no.	
	I	II
Energy gain from 15 per cent protein greater than from 10 per cent protein	27:1	49:1
Energy gain from 20 per cent protein greater than from 10 per cent protein	3332:1	1110:1
Energy gain from 25 per cent protein greater than from 10 per cent protein	.....	88:1
Energy gain from 20 per cent protein greater than from 15 per cent protein	18:1	2:1
Energy gain from 25 per cent protein greater than from 15 per cent protein	53:1	3:1
Energy gain from 25 per cent protein greater than from 20 per cent protein	2:1	2:1



The digestibility of both the energy producing nutrients (table 3 and figs. 3 and 4) and the protein of the diets (table 3 and figs. 5 and 6) increased slightly but consistently with increase in the plane of protein intake.

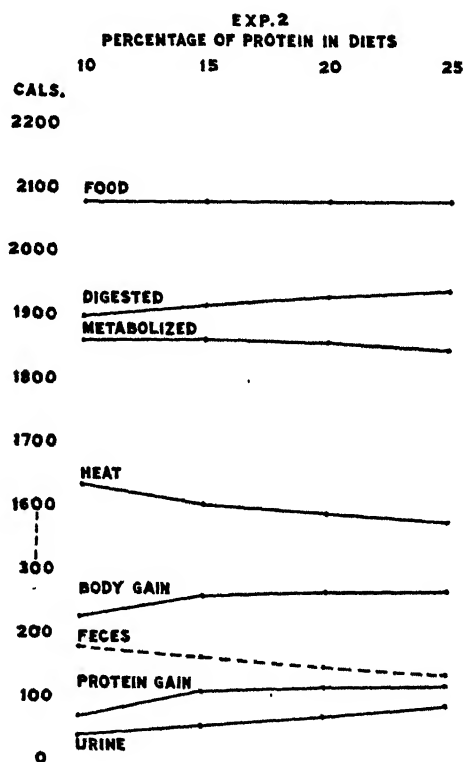


Fig. 4 Experiment 2. Average utilization of food energy during 10 weeks as affected by differences in the plane of protein intake.

The metabolizable energy of the diets, however, was essentially the same at all planes of protein intake (figs. 3 and 4), since the urinary energy increased almost exactly in proportion as the feces energy decreased, with rise in proportion of protein in the diets.

Data relating to the effects of the plane of protein intake on the composition of the body gain are given in table 4.

TABLE 3  
Average digestibility of nitrogen and energy producing nutriment

PLANE OF PROTEIN INTAKE	NITROGEN		ENERGY	
	Feces	Digested	Feces	Digested
	<i>gm.</i>	<i>per cent</i>	<i>Cal.</i>	<i>per cent</i>
Experiment no. 1				
10% protein	0.88	88.1 $\pm$ 0.24	184.4	91.5 $\pm$ 0.17
15% protein	0.90	91.8 $\pm$ 0.13	178.0	91.8 $\pm$ 0.26
20% protein	0.97	93.3 $\pm$ 0.10	152.7	92.9 $\pm$ 0.07
25% protein	1.05	94.2 $\pm$ 0.16	143.0	93.4 $\pm$ 0.09
Experiment no. 2				
10% protein	0.83	88.3 $\pm$ 0.14	178.4	91.4 $\pm$ 0.13
15% protein	0.90	91.5 $\pm$ 0.12	162.0	92.2 $\pm$ 0.12
20% protein	0.95	93.2 $\pm$ 0.08	147.7	92.9 $\pm$ 0.06
25% protein	0.96	94.6 $\pm$ 0.13	138.2	93.3 $\pm$ 0.06

NOTE: Each datum is an average value representing twelve animals on a continuous metabolism investigation during 10 weeks.

TABLE 4  
Average nitrogen of body gain related to fat and energy of body gain and to nitrogen of feed

PLANE OF PROTEIN INTAKE	NITROGEN OF BODY GAIN	FAT GAINED		ENERGY GAINED			NITROGEN OF FEED	
			Per gram nitrogen gained	Total	As protein	As fat		Utilized for body gain
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>Cal.</i>	<i>Cal.</i>	<i>Cal.</i>	<i>gm.</i>	<i>per cent</i>
Experiment no. 1								
10% protein	2.51	13.9	5.5 $\pm$ 0.18	224.2	93.4	130.8	7.41	33.9 $\pm$ 0.58
15% protein	3.68	13.3	3.6 $\pm$ 0.26	262.7	137.4	125.3	11.04	33.3 $\pm$ 0.63
20% protein	4.13	15.2	3.7 $\pm$ 0.24	295.9	152.6	143.3	14.49	28.5 $\pm$ 0.62
25% protein	4.17	15.4	3.7 $\pm$ 0.25	301.6	156.4	145.2	18.17	22.9 $\pm$ 0.60
Experiment no. 2								
10% protein	1.91	16.8	8.8 $\pm$ 0.45	226.4	68.4	158.0	7.05	27.1 $\pm$ 0.57
15% protein	3.02	16.1	5.3 $\pm$ 0.33	259.4	107.7	151.7	10.53	28.7 $\pm$ 0.58
20% protein	3.29	15.9	4.8 $\pm$ 0.34	267.0	117.2	149.8	14.06	23.6 $\pm$ 0.68
25% protein	3.42	15.7	4.6 $\pm$ 0.35	270.2	121.5	148.7	17.59	19.4 $\pm$ 0.60

NOTE: Each datum is an average value representing twelve animals on a continuous metabolism investigation during 10 weeks.

With increase in the proportion of protein in the diets there were invariably increases, in the same order, in the nitrogen and energy gained, and usually a decrease in the percentage of food nitrogen utilized for body gain—signifying diminishing necessity for economy in nitrogen utilization.

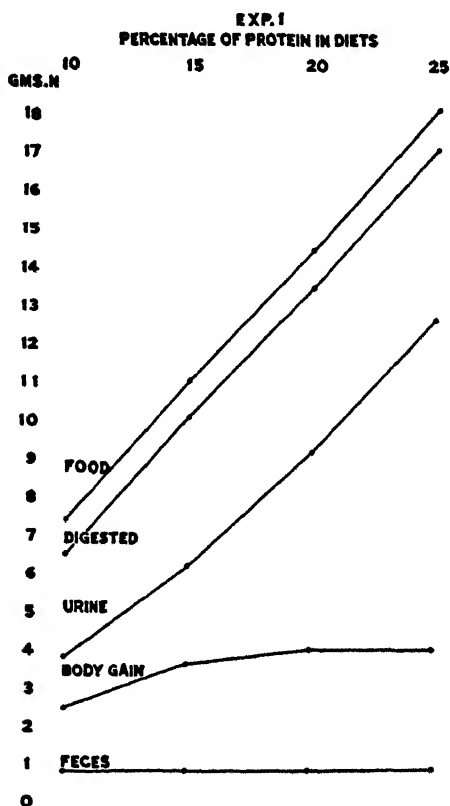


Fig. 5 Experiment 1. Average utilization of food nitrogen during 10 weeks as affected by differences in the plane of protein intake.

This situation is graphically represented in figures 5 and 6 by the curves showing that with increase in the protein contents of the diets the nitrogen retained as body gain increased at a decreasing rate, and the nitrogen of the urine increased at an increasing rate.

The curves representing feces nitrogen, in figures 5 and 6, show that with increase in the protein of the diets the feces nitrogen increased but little, because of the fact that the protein of the diets was mainly in the form of casein, which was highly digestible.

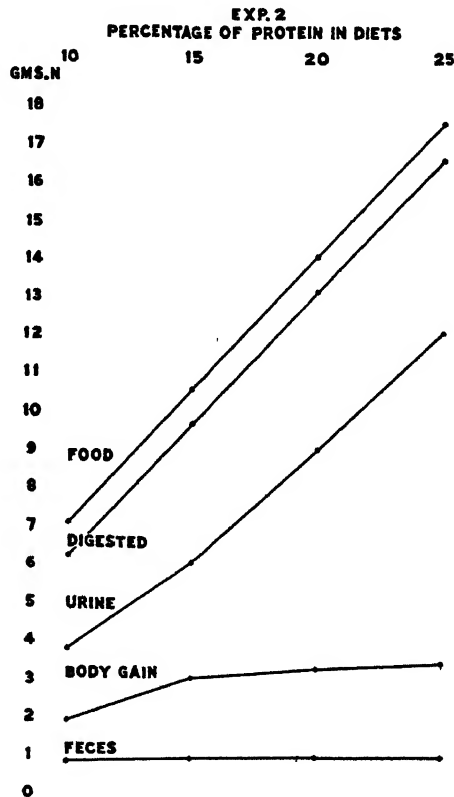


Fig. 6 Experiment 2. Average utilization of food nitrogen during 10 weeks as affected by differences in the plane of protein intake.

The relation of the protein content of the diets to the heat loss is shown in table 5, and in figures 3 and 4.

The first thought that naturally comes to mind in this relation is that with increase in the protein content of these equicaloric diets the specific dynamic effect of the protein would have the effect to increase the heat production. This, however, is incorrect. The results of these experiments show

clearly that increases in the protein content of equicaloric diets which improve their nutritive balance, as indicated by increased retention of protein and energy, and increased gain in body weight, have the effect to diminish rather than to increase the heat production.

The heat production was measured indirectly, as the difference between the gross energy of the food and the combined values for the energy of the excreta and of the body increase, in one value representing the entire 10-week period of experimentation.

TABLE 5  
*Average heat loss related to energy of food*

PLANE OF PROTEIN INTAKE	FEED ENERGY	HEAT LOSS	
			Per cent of feed energy
	<i>Cal.</i>	<i>Cal.</i>	<i>per cent</i>
Experiment no. 1			
10% protein	2163.6	1708.5	79.0 $\pm$ 0.31
15% protein	2163.6	1668.2	77.1 $\pm$ 0.42
20% protein	2163.6	1642.7	75.9 $\pm$ 0.46
25% protein	2163.6	1626.2	75.2 $\pm$ 0.43
Experiment no. 2			
10% protein	2075.9	1631.9	78.6 $\pm$ 0.41
15% protein	2075.9	1600.6	77.2 $\pm$ 0.41
20% protein	2075.9	1590.4	76.6 $\pm$ 0.40
25% protein	2075.9	1577.7	76.1 $\pm$ 0.42

NOTE: Each datum is an average value representing twelve animals on a continuous metabolism investigation during 10 weeks.

The energy of the body increase was also determined indirectly, by difference between the energy of the bodies of the rats at the end of the experiment and of a control group which was carefully selected to represent the experimental groups at the beginning of the study.

In spite of the fact that this measurement of the heat production was indirect, and determined by difference, as explained, it is the judgment of the writers that the values so determined are more accurately representative of the heat production of the animals than any measurement covering only a few minutes or a few hours could possibly be.

These indirect heat measurements are relatively large values, in comparison with those representing the excreta and the body gain, and inasmuch as they represent the total heat production for a 10-week period, they should be free from such extensive errors as would be inevitable if the heat were determined directly, in short periods of observation, and were then multiplied by the large numbers required to render them representative of the whole period of experimentation.

The reliability of these indirect heat measurements is strikingly shown by the close agreement of the curves of heat production for the two experiments (figs. 3 and 4).

As an indication of the accuracy of the operations involved in this indirect heat measurement—in the first experiment the average percentage recovery of feed nitrogen, in feces, urine and body gain—was 98.3 per cent, for the forty-eight rats, with a coefficient of variation of only 1.51 per cent.

The difference between 98.3 per cent and 100 per cent recovery of nitrogen represents experimental error, including shed hair, and ammonia vaporized from the excreta.

A noteworthy arithmetic consequence of the method used of accounting for the food energy is that with equicaloric diets of essentially the same metabolizability, as in these experiments, the energy of the body gain and the heat production can only vary reciprocally—because, jointly, they complete the total.

Duplicate determinations of the energy of materials, by means of the bomb calorimeter, were accepted only when the variation was within 1 per cent, except with urine, in which case a 2 per cent variation was accepted. The duplicate analyses of the forty-eight samples of feces differed, on the average, by 0.30 per cent, and the corresponding values for ether extracts of the rat bodies differed by 0.25 per cent.

The average of the duplicate energy determinations entering into the measurement of the heat production, therefore, are believed to be not more than  $\frac{1}{2}$  of 1 per cent in error.

The odds relating to the heat production as affected by the percentage of protein in the diets were as follows:

	<i>Experiment no.</i>	
	<i>I</i>	<i>II</i>
Heat production from 10 per cent protein greater than from 15 per cent protein	48: 1	26: 1
Heat production from 10 per cent protein greater than from 20 per cent protein	1110: 1	3330: 1
Heat production from 15 per cent protein greater than from 20 per cent protein	19: 1	2: 1
Heat production from 15 per cent protein greater than from 25 per cent protein	224: 1	13: 1
Heat production from 20 per cent protein greater than from 25 per cent protein	4: 1	4: 1

A detail of importance in the conduct of this investigation was the method of control of food consumption—especially the prevention of waste of food and of contamination of excreta by waste food.

This was accomplished by means of a holder for the feed cup, made of galvanized iron and illustrated in figure 7. This apparatus, which was devised by R. W. Swift, is 5 inches wide and  $3\frac{1}{2}$  inches from front to back, the back being  $3\frac{3}{8}$  inches high, and the front  $1\frac{3}{16}$  inch high. The feed cup itself was a 50 cc. beaker.

The device was designed for use in the cylindrical cage illustrated in this journal, vol. 8, August, 1934, p. 206. This illustration, however, does not show the inverted pie-tin which serves as a cover for the cage.

To save floor space within the cage the feeder is elevated by suspension from the top of the cage, by means of the hook shown in the figure.

The use of a beaker as a feed container makes for convenience in weighing, handling and cleaning.

The beaker is held firmly in place in a metal cup, and the surrounding waste pan catches food thrown out of the beaker. Even during long periods of use, the amount of feed thus scattered is negligible. A loose metal ring, with cross wires, is sometimes placed on top of the feed in the beaker, to prevent the rat from scooping out the feed, but the use of this accessory is rarely required.

The top of the feeder is covered by a loose piece of  $\frac{1}{2}$ -inch-mesh galvanized wire screen, hinged at the back, and with an opening at the center to permit access of the rat to the beaker. In cutting this opening the wires at the back are bent down, to the edge of the beaker, and the wires at the sides are bent vertically upright to discourage the rat from sleeping on this screen. If the rats learn to raise this hinged screen it may be fastened down with a wire hook.

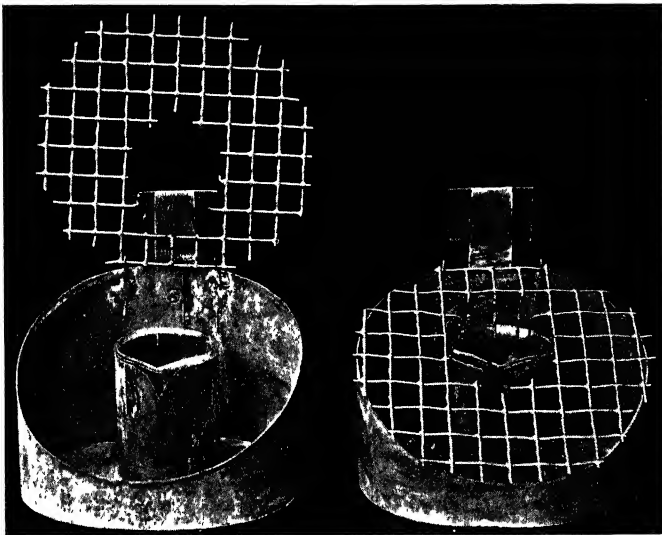


Fig. 7 Feeding cup for use in nutrition experiments with the rat.

The purpose of this screen cover is to prevent the access of the rat to feces dropped in the waste pan surrounding the beaker, though in most cases no feces are dropped there.

When this device is used with very unpalatable rations small quantities of food may be thrown outside the apparatus, but this happens much less frequently than in the use of several other types of feeders with which it has been compared.

The method of the investigation did not provide for the complete factoring of the heat production. Under the conditions existing, however, it was practicable to make a series



of heat measurements of each of the forty-eight rats utilized in the second experiment, which may be considered in the general light of measurements of basal metabolism, though basal conditions as usually defined were not maintained with exactness.

The rats being fed once per day, one measurement of the approximate basal heat production of each individual was made by the open-train Haldane procedure. Thirty-six of these measurements were made during the seventeenth to twenty-third hours and twelve during the twenty-fourth to thirtieth hours after feeding. In each case the rat was in the chamber for 7 hours, but the heat production of the first hour was rejected on account of voluntary movements of the animals when first put into the chamber.

The time at which the rats finished eating was not observed. These measurements, therefore, do not exactly represent the basal metabolism, as usually defined, though they do so in an approximate manner.

Thirty-two of the rats were subjected to this measurement during the seventh week of experimentation, and the remaining sixteen during the ninth week. The quadruplets were handled as nearly alike as possible, two animals on one day, and the other two on the following day.

The procedure followed was the same as that described by Forbes, Kriss and Miller ('34) except that the water bath was operated as an air bath, in order to avoid the necessity of drying the respiration chamber (bottle) before weighing. With this change, the rat, which apparently slept during the 6-hour respiration measurement, under the influence of the bright light, usually remained asleep while it was weighed at the end of this period.

These approximate basal heat determinations, given in table 6, show that the plane of protein intake was without appreciable effect on this measurement. The difference in the heat production of the four groups of rats, resulting from the difference in protein intake and as measured for the 10-week period, therefore, represented either physical activity, or dynamic effect, or both.

A computation of the greatest possible differences in the dynamic effects of the diets employed which might be expected in the light of the separate determination of the specific dynamic effects of protein, carbohydrate and fat, by Kriss, Forbes and Miller ('34) indicates that in all probability these differences are so small as to lie within the range of the inevitable errors of the most accurate measurements of such values that are now attainable. It is the judgment of the writers, therefore, that the differences in heat production of the rats in these experiments which are responsible for the falling curves of heat production coincident with increase in

TABLE 6

*The approximate basal heat production of albino rats which received equicaloric diets containing different percentages of protein*

PROTEIN IN DIET	BASAL HEAT PER HOUR	LIVE WEIGHT	SURFACE AREA	BASAL HEAT PER SQUARE CENTIMETER PER HOUR	NON-PROTEIN R. Q.
<i>per cent</i>	<i>Cal.</i>	<i>gm.</i>	<i>sq.cm.</i>	<i>Cal.</i>	
10	593.0	99.8	198.5	3.0	0.74
15	658.6	121.3	223.1	3.0	0.75
20	701.8	132.2	235.0	3.0	0.75
25	690.1	135.0	238.0	2.9	0.76

NOTE: Each datum is an average value representing twelve animals.

protein intake are primarily and almost exclusively expressions of differences in voluntary activity—for which there are no accurate quantitative measures, separate from the dynamic effects of the diets.

Surface area, as given in table 6, was computed by the use of the formula—surface area, in sq.cm., =  $12.54 W$  (gms.)<sup>0.6</sup>, Lee ('29).

With diets of the same energy value, therefore, differences in the plane of protein intake influence the food utilization of the growing rat in a complication of ways including, most notably, the amount and composition of the body increase, the heat loss and the efficiency with which both food energy and food nitrogen were digested and were retained as growth.

The degrees of the observed effects obviously apply with exactness only to growing rats, on the particular diets fed; but other animals, on other diets, undoubtedly respond in similar ways to similar influences.

#### SUMMARY

The effects of four planes of protein intake (10, 15, 20 and 25 per cent) were studied by means of two 10 weeks' growth, metabolism and body analysis experiments on forty-eight albino rats each. The rats for each experiment were selected as twelve groups of four individuals, each such group being of one sex and of the same litter, and each rat in each such group received the same energy but different protein intake. There were, therefore, twelve rats on each treatment in each experiment.

The effects of the increasing protein content of the equicaloric diets were as follows:

Increase in gain in body weight, at decreased cost in terms of dry matter of food; increase in efficiency of digestion and retention of protein and of energy-producing nutriment; increase in urinary nitrogen at an increasing rate, and increase in protein of the body at a decreasing rate; increase in energy of the urine coincident with decrease in the energy of the feces, the metabolizable energy, therefore, remaining practically constant; diminished efficiency in the utilization of food nitrogen; no regular change in amount of fat gained, but usually a decrease in fat gained in proportion to protein gained.

Increases in the protein of equicaloric diets having the effect to improve their nutritive balance made no change in the basal heat production per unit of computed surface area, but diminished the total heat production of the animals, as they lived under normal conditions of freedom of activity.

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# PATHOLOGICAL SKIN CHANGES IN THE TAIL OF THE ALBINO RAT ON A DIET DEFICIENT IN VITAMIN G<sup>1, 2</sup>

SUSAN GOWER SMITH AND DOUGLAS H. SPRUNT

*Departments of Physiology and Pharmacology and Pathology, Duke University  
School of Medicine, Durham, North Carolina*

SIX FIGURES

(Received for publication July 19, 1935)

In the course of certain vitamin G studies it was noticed by one of us (Smith, '32) that the sebaceous glands in the tails of the deficient rats were atrophied. We have now been able to confirm these findings and to show that the change is independent of the initial age of the rat and the season of the year. The sebaceous gland change is regularly accompanied by what appears to be altered secretion which is interesting in view of the fact that abnormal secretion has been reported in cases of human pellagra (Corkill, '34).

## EXPERIMENTAL

*Animals.* White albino rats of The Wistar Institute strain were used in this work with the exception of thirteen of the controls used in experiment I. The latter were obtained locally and were of unknown, but probably poor nutritional background. Approximately equal numbers of males and females were used, and their weights were recorded weekly.

<sup>1</sup> Vitamin G(B<sub>2</sub>) as used in this paper includes the factors of the vitamin B complex which withstand autoclaving.

<sup>2</sup> Part of the expenses of this work was covered by a grant from the Lederle Laboratories, Pearl River, New York.

The rats were divided into groups of six in cages with raised screen bottoms (1.25 cm. mesh) to minimize the effect of coprophagy. They had access to the diet and to fresh distilled water at all times.

*Diet I.* This is a stock diet which has proven biologically adequate in our colony. It consists of 'Bal Ra,' a commercial dog food, supplemented with cod liver oil at a level of 2 per cent and brewer's yeast at a level of 10 per cent.

*Diet II.* This diet was designed to approximate the one on which the 'poor whites' in the south develop pellagra and is similar to the one on which Goldberger ('15) produced pellagra in human subjects in his prison farm experiment. Its composition is as follows: corn meal (white, water ground) 50 per cent; pork fat (or salt pork) 20 per cent; Brer Rabbit molasses (Gold Label) 15 per cent; white wheat flour 10 per cent; cane sugar 5 per cent.

*Diet III.* This is a vitamin G deficient diet of Bourquin and Sherman ('31). It consists of purified casein 18 per cent; butter fat 8 per cent; Osborne and Mendel's ('13) salt mixture 4 per cent; cod liver oil 2 per cent; cornstarch 68 per cent. A part of the cornstarch carries the strong 80 per cent alcoholic extract from 50 gm. of whole wheat for each 100 gm. of diet as the source of B in the diet.

*Diet IV.* This is identical with diet III except that yeast autoclaved in thin layers  $2\frac{1}{2}$  hours at 15 pounds pressure is introduced at a level of 10 per cent, replacing an equal weight of cornstarch.

*Diet V.* This is the same as diet III except that dried egg white is introduced at a level of 10 per cent, replacing an equal weight of cornstarch (Chick and Roscoe, '29).

The egg white used as a supplement in diet V was coagulated by boiling for 30 minutes in an aluminum double boiler stirring constantly to break up the particles. The moist coagulated product was then dried in thin layers on glass dishes at a temperature from 80 to 90°C. for 2 hours and was allowed to stand over night in the oven.

*Method of sectioning tail.* For biopsy a portion of the tail approximately 0.5 cm. long was cut from the distal end with a very sharp razor blade and fixed in 10 per cent formalin.

On necropsy three sections were made, one from the tip, one from the mid-portion, and one from the base and similarly fixed. The sections were decalcified in equal parts of 85 per cent formic acid and 20 per cent sodium citrate, embedded in paraffin and then sectioned and stained with hematoxylin and eosin.

Table 1 shows the general plan of the experiment. A biopsy was made whenever the diet was changed. Sections were also made when the animal died or was sacrificed.

*Results.* a. Clinical. Almost immediately after placing the animals on the deficient diets there was the usual slowing of the growth rate as illustrated for experiment III in figure 1. The life spans in experiment I varied from 70 to more than 225 days except for diet II where all were dead in 175 days. This could not be observed in the other experiments because the animals were sacrificed too soon.

Tail lesions appeared in 70 to 90 days on diets II and III. These usually consisted of a dry scaliness beginning at the tip and extending up the tail together with a dry yellow waxy coating resembling hardened sebaceous secretion (fig. 2). With the older animals of experiment I and particularly on diet II the lesions differed somewhat in being darker and more greasy, possibly contaminated with food and dirt over the longer period of time. This did not occur on the adequate diet I and disappeared gradually when autoclaved yeast was fed, the tail finally becoming quite clean and exhibiting a pink appearance as of new tissue.

One of the first symptoms observed was a staining of the wrists and forepaws with a dark red material giving a positive guaiac and benzidine test for blood. This staining had a tendency to come and go, but it appeared at sometime in all the deficient rats except one. Priapism also occurred very frequently. Reproduction did not occur on any of the deficient diets. General alopecia was fairly constant and was manifested by the disappearance of the long coarse hairs so that



TABLE 1

EXPERIMENT	SEASON	GROUPS	NUMBER OF RATS	DIET	AGE AT START OF EXPERIMENT	INITIAL WEIGHT	MAXIMUM NUMBER OF DAYS ON DEFICIENT DIET	NUMBER OF SURVIVORS AT END OF PERIOD	FATE AT THIS TIME
I	Oct. 7, 1931 to Sept. 7, 1932	I	19	I	days			3 (others killed at intervals for comparison)	Killed
		II	17	II	45	74	226	0	.....
		III	18	III				6	Diet IV. 1 died 1 day later. 1 died 6 days later
II	July 19, 1933 to Dec. 5, 1933	I	6	III				4	4 continued 36 days
		II	2	VI	23	38	112	1	2 killed 1 died 1 diet IV 36 days. Killed
III	May 1, 1934 to Sept. 1, 1934	I	10	III			70	10	Killed
		II	15	III			90	15	Killed
		III	15	III			100	15	Killed
		IV	4	III	27-30	56	84	4	Diet IV (6, 9, 15, 16 days)
		V	4	III			90	4	Diet IV (36 days)
		VI	12	III			90	12	Diet V (36 days)
		VII	11	IV			90	10	Killed

the coat became fuzzy and soft like that of a much younger rat. Xerophthalmia occurred regularly on diet II.

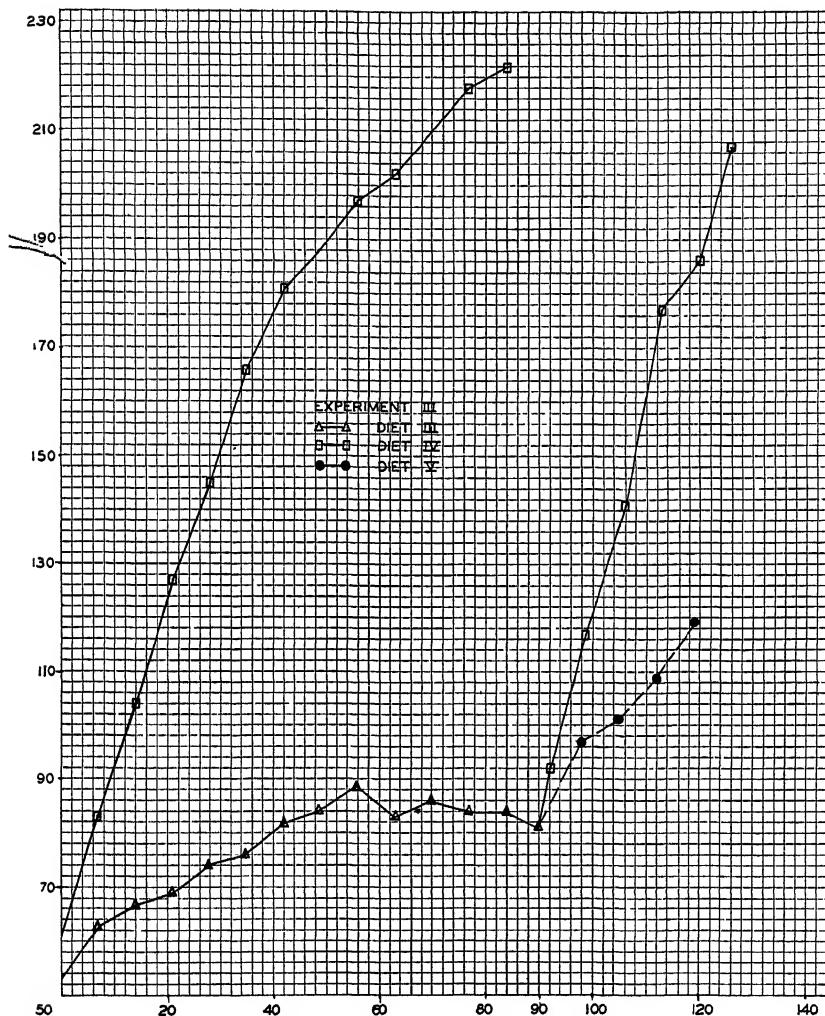


Fig. 1 Growth curve of rats in experiment III.

Other symptoms such as dermatitis of the digits described by György ('35) occurred occasionally. Contrary to the findings of Day, Langston and O'Brien ('31) cataract did not

occur except in one case and that only after autoclaved yeast had been added to the diet.

b. Microscopic. At 70 days there were only slight changes in the epithelium and sebaceous glands of the tail. After 90 days the epithelium becomes thinner and the amount of keratinized material much less. The growth of the basal cells appears to be greatly reduced, probably accounting for the decrease in keratin. The sebaceous glands in the earlier stages may show only a decrease in the amount of cytoplasm



Fig. 2 Gross appearance of tails. A. Photograph of tail of control rat on diet IV, experiment III. B. Photograph of tail of deficient rat after 100 days on diet III, experiment III.

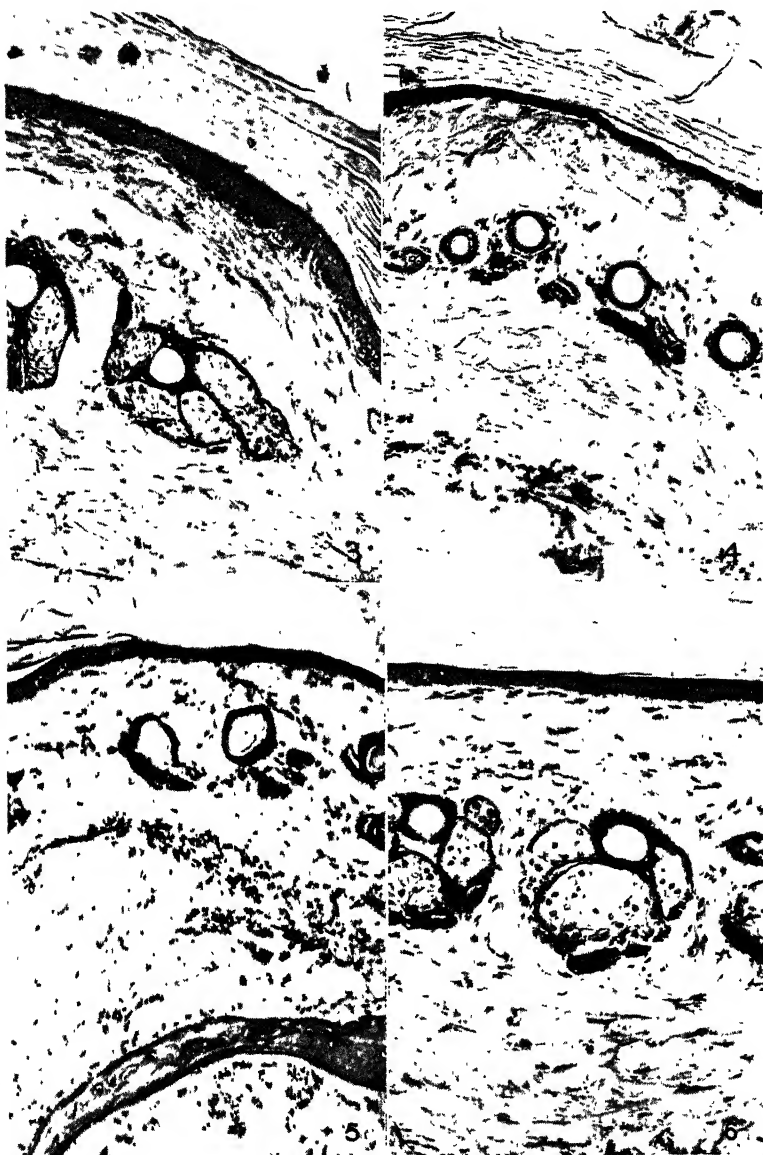
but later the number of cells decreases and in some of the rats killed after 100 days they are represented only by a few cells with very little cytoplasm. The fat which in the normal surrounds the glands is replaced by connective tissue having

Fig. 3 Photomicrograph of cross section of the tail of control rat on diet IV, experiment III. Note sebaceous glands, epithelium and keratin.  $\times 150$ .

Fig. 4 Photomicrograph of cross section of the tail of a deficient rat on diet III for 100 days in experiment III. Note extensive atrophy of sebaceous glands and epithelium and also the decrease of keratin.  $\times 150$ .

Fig. 5 Photomicrograph of cross section of the tail of a deficient rat on diet III for 90 days in experiment III. Note atrophy of sebaceous glands and epithelium.  $\times 150$ .

Fig. 6 Photomicrograph of the cross section of the same rat as shown in figure 5, 36 days after autoclaved yeast had been added to the diet. Note the almost complete return to normal of the sebaceous glands and epithelium.  $\times 150$ .



Figures 3 to 6

a peculiar hyalinized appearance. There is no round cell infiltration, accumulation of polymorphonuclear leucocyte or any other change which would indicate an infection.

Regeneration occurs when the diet is supplemented with autoclaved yeast (diet IV). This was not evident in two rats after 6 days on the supplemented diet, was definite but slight in one rat after 9 days, was more marked in two rats after 15 and 16 days, respectively, and apparently complete in four rats after 36 days.

Some regeneration occurred in rats given egg white (diet V) but this was less marked just as their growth rate was slower than in the case of rats receiving the autoclaved yeast.

*Control experiments.* In order to see whether the changes described above were due to inanition or were specific for vitamin G the following experiments were carried out.

1. Eight rats were placed on Steenbock and Black's ('25) rickets producing diet no. 2965 at the age of 28 days and maintained on it for 66 days when rickets could be demonstrated both clinically and by roentgenogram. Cross sections of the tails revealed practically normal sebaceous glands.

2. Ten rats were deprived of food but given water. This resulted in gradual loss of weight, general weakness and death, but no characteristic lesion. Microscopic preparations of the tails of these rats show a replacement of the fat around the sebaceous glands with connective tissue but no change in the epithelium or sebaceous glands themselves.

3. Twelve rats were given food but deprived of water. These also lost weight and died, but in addition all the rats developed the blood stained wrists often described as a symptom in vitamin G deficiency (Chick and Roscoe, '28; Kou, '31; Akroyd, '30; Sherman and Derbigny, '32). The mouth, nose and eyes were also stained. Microscopic preparations of the tails of these rats were essentially the same as those deprived of food.

4. Ten rats were placed on Sherman's A free diet (Sherman and Smith, '31) at the age of 28 days and maintained on it for 66 days. At this time they all showed xerophthalmia,

the weight was decreasing rapidly, one died on the sixty-sixth day and the others were obviously moribund. Abscesses were present at the base of the tongue at autopsy in all ten rats. Microscopic preparations of the tails revealed a marked atrophy of the epithelium, the sebaceous glands showed a slight decrease in the size of the cells and in some instances they were represented by only a few small cells. The fat around these glands had disappeared, its place being taken by young connective tissue.

5. Ten 28-day-old rats were placed on a B deficient diet identical with that of Chase and Sherman ('31) except that the vitamin G was supplied by brewers' yeast autoclaved  $2\frac{1}{2}$  hours at 15 pounds pressure instead of autoclaved baker's yeast. They were maintained on it until they died, all showing the characteristic neuromuscular changes before death. The microscopic tail sections showed essentially the same thing as those of the A-deficient rats with the exception that the sebaceous gland change was more and the epithelial change less marked.

#### DISCUSSION

We have presented evidence to show that an atrophy of the sebaceous glands and thinning of the epithelium together with hyalinization of the connective tissue occurs in the tails of rats subsisting on a diet deficient in the vitamin G complex. This does not occur in rats on the same diet supplemented with autoclaved yeast. After the atrophy has occurred in any vitamin G deficient rat, as demonstrated by biopsy section, there is prompt and complete regeneration upon the addition of autoclaved yeast to the diet. However, we cannot say that this characteristic change is specific for vitamin G as it also occurred to some extent in the rats on vitamin A deficiency and in the rats on vitamin B deficiency in our control experiments. There are three possible explanations for the pathological changes observed 1) they may be due to chronic inanition, 2) in the case of the vitamin A and vitamin B deficiencies there may be a secondary shortage of vitamin G

due to inanition or faulty absorption, 3) vitamins A, B and G may all be necessary for the protection of the tissues involved. Under these circumstances the pathological changes described may occur when any one of them is lacking. Experiments are now in progress to test these hypothetical explanations.

It should be emphasized that in the rats deprived of water, the blood stained wrists occurred regularly and became gradually worse until the rat died. This is often described as a symptom of vitamin G deficiency, but it also occurs in other deficiencies and frequently accompanies infections. It is non-specific and apparently results from dehydration.

It is impossible to state what factor or combination of factors is responsible for maintaining the characteristic structure of the sebaceous glands and epithelium. The fact that abnormal sebaceous secretion has been reported in cases of human pellagra (Bigland, '20; Biggam and Ghalioungui, '33; and Corkill, '34) and the results of the present studies lend some support to the idea of vitamin G deficiency. Corkill made his observations on Sudanese millet eaters which would preclude any effect due merely to corn meal. He states that in pellagra the sebaceous glands hypertrophy. There was no histological evidence presented for this, however, and the subsequent observation that there is a 'yellowish secretion' which has been aptly likened to 'sulfur flakes' indicates an abnormal secretion. We attempted to determine by means of bi-weekly biopsies on the tails of rats whether or not an hypertrophy preceded the atrophy of the sebaceous glands. This experiment was inconclusive, however, since the bleeding and the infection obscured both the gross and microscopic results. We have observed engorged sebaceous glands palpable to the touch in pellagra patients in this hospital and have also seen the 'sulfur flake' type of secretion.

No attempt has been made to differentiate the factors of the vitamin G complex as described by the English investigators (György, '35 confirmed by Harris, '35 and Chick, Copping and Edgar, '35). The fact that we did not get the characteristic dermatitis of the digits regularly may be explained by the

possible contamination of either the cornstarch (Hogan and Richardson, '34) or the source of B with the protective factor.

#### SUMMARY AND CONCLUSIONS

1. Seventy-four rats given Bourquin and Sherman's G-deficient diet for 90 days or longer showed varying degrees of atrophy of the sebaceous glands and thinning of the epithelium in cross sections of the tail. The fat normally present around the sebaceous glands was replaced by connective tissue. There was no cellular infiltration.

2. The positive control rats, thirteen receiving the same G-deficient diet, supplemented with autoclaved yeast and nineteen receiving the stock diet had normal sebaceous glands and epithelium.

3. After the change in the sebaceous glands and epithelium was produced in sixteen rats as shown by biopsy, autoclaved yeast was added to the diet. In every case where the rat lived longer than 9 days there was evidence of regeneration and by the end of 36 days it was practically complete, the sebaceous glands and epithelium appearing normal.

4. No sebaceous gland or epithelial changes were noted in ten rats deprived of food nor in twelve rats deprived of water. The latter, however, had the blood stained wrists so often described as a symptom in vitamin G deficiency.

5. In the vitamin control experiments some changes in the epithelium and sebaceous glands were noted in vitamin A deficiency and in vitamin B deficiency. There was no appreciable change in the rats on Steenbock's rickets producing diet.

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## HUMAN IODINE BALANCE

VERSA V. COLE AND GEORGE M. CURTIS

*Department of Medical and Surgical Research, Ohio State University,  
Columbus, Ohio*

NINE FIGURES

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In comparison with the work on other phases of iodine metabolism, there is a paucity of work on iodine balance in human subjects. The only long study which we have available on a single individual is that of von Fellenberg on himself ('26). He analyzed urine, stool, nasal secretion and sweat. His studies were carried out consecutively on four levels of intake: 17, 55, 75 and 80 micrograms of iodine per day. The basal diet was the same throughout. The iodine was added in various forms as indicated. Complete studies were made for 6 days on the 17 micrograms intake after he had been on the diet for 21 days. The average total excretion per day was 14.2 or 84 per cent of that ingested, the nasal and sweat secretion accounting for about 15 per cent. For 7 days immediately following this he increased his intake to 55 to 58 micrograms of iodine per day by adding KI. Complete analyses were reported for the last 6 days. The average daily iodine output was 38 micrograms per day or 67 per cent of that ingested. Ten per cent was accounted for in the sweat and nasal secretion. Twenty-four micrograms of this was in the urine. By replacing the KI with cod liver oil the iodine intake was raised to 75 micrograms per day for 6 days. The average daily iodine excretion was 37 micrograms per day or only 50 per cent of that ingested. The distribution of the excreted iodine was the same as that in the period preceding.

Immediately following this for 5 days sardines or watercress were added to the diet to make the iodine content 77.5 to 80 micrograms of iodine per day. The excretion of iodine through the nasal secretion and sweat remained unchanged. The urinary iodine varied from 21 to 58 and the total excretion from 36 to 97 with an average of 63 micrograms of iodine or 80 per cent of that ingested. Following this increased iodine feeding the intake was decreased at once to the original level and the results for 5 days reported. The average iodine output per day was 38 micrograms and the output was as great on the fifth day as on the first. From these data several facts seem evident. Sweat and nasal iodine excretion were uninfluenced by changes in the iodine intake, stool iodine was only slightly altered. The iodine in KI is excreted more rapidly than the iodine in cod liver oil, sardines or watercress. When the intake of iodine was suddenly decreased the subject was not approaching balance at the end of 5 days.

Scheffer ('33 a, '33 b) carried out balance studies on twelve normal subjects with balance periods of 3 or 5 days. He analyzed urine, stool and sweat. Intake levels studied were 54, 59, 66, 130 and 155. In two patients with intakes of 54 micrograms of iodine per day the total outputs were 53 and 49. Of this, 18 and 29 were in the sweat. Then in these two subjects 98 and 90 per cent of the intake was accounted for through excretion. The combined urine and stool output accounted for only 65 and 37 per cent. One subject excreted 28 in the urine, 3.1 in the stool, and 10 in the sweat. Only 53 per cent of the intake was accounted for in the urine and stool combined. The iodine excretion through urine and stool was reported on seven individuals whose intakes varied from 62 to 70 micrograms of iodine per day. The combined excretion in these individuals varied from 21 to 28 micrograms of iodine per day or an excretion of 30, 33, 34, 34, 37, 42 and 44 per cent of that ingested. One subject with an intake of 130 micrograms per day excreted 57 in the urine, 2.8 in the stool and 45 through the skin. The combined excretion was 105 or 81 per cent of that ingested. The urine and stool accounted for

46 per cent of that ingested. Another normal subject with a daily intake of 155 micrograms excreted 116 micrograms in the urine and 24 in the sweat giving a combined excretion of 140. Seventy-five per cent of that ingested was accounted for in the urine alone. The fecal iodine was mentioned in only a few instances because of its constancy regardless of intake. The fecal iodine varied from 2 to 10 micrograms per day with an average of less than 5 micrograms per day. The same was true of the fecal iodine in von Fellenberg's study.

Attention is called to fecal iodine at this time because it is exceedingly important in the hyperthyroid patients as shown by Scheffer's work ('33, '34). Scheffer carried out three studies on subjects with goiter without hyperthyroidism, seven on subjects with goiter and hyperthyroidism, and six on subjects with Basedow's disease. With goiter, with or without hyperthyroidism, the iodine output in the urine was decreased. In goiter alone, the iodine output through the lungs was increased. In hyperthyroidism, with or without goiter, the fecal iodine was increased to several times the normal value. In Basedow's disease the iodine output through the skin was also increases.

In the three cases of goiter without hyperthyroidism the results were as follows: with an intake of 80 micrograms of iodine per day, there was 15 in the urine, 5 in the stool, and 40 through the lungs, giving a total excretion of 60 micrograms per day or 75 per cent of the intake; with 105 ingested, 9 micrograms per day was excreted in the urine, 9.5 in the stool, and 15 through the lungs, total 33 micrograms of iodine per day or 31 per cent of that ingested; with an intake of 139, excretion in the urine was 14.6, in the stool, 4.7, and through the lungs, 30 micrograms of iodine per day, totaling 49 micrograms or 35 per cent of that ingested.

In hyperthyroidism with goiter, urine and stool iodine are reported on seven cases of known intake; sweat excretion on three, and lung excretion on one. Four patients with intakes of 80 to 100 micrograms per day excreted from 2.5 to 9.0 micrograms per day in the urine, while a fifth on the same

intake excreted 25. The five excreted from 33 to 68 micrograms per day in the stool. The excretion through the skin in the three cases studied was 2.1, 14 and 16 micrograms per day. The total excretion varied from 40 to 91 with an average of 70 micrograms per day. One patient with an intake of 100 to 120 eliminated 16 in the urine, 67 in the stool, 27 in the sweat, and 17 in the expired air. Total excretion was 127 micrograms per day. Another with an intake of 150 excreted 94 in the urine, 35 in the stool, and 20 in the sweat, making a total of 149.

In Basedow's disease with intakes varying from 75 to 110 the urinary excretion varied from 23 to 58 in four cases and was 265 in the fifth. Stool iodine varied from 24 to 61 in four cases and was 7 in another case. Iodine excretion through the skin in two cases was 216 and 109. Combined excretions varied from 119 to 289 with the exception of one case which excreted 69. The average of all the combined excretions was 184. One patient with an intake of 130 excreted 26 in the urine, 49 in the stool, and 53 in the sweat, giving a total of 128. These results will be discussed further in connection with our own.

#### PROCEDURE

Our investigations on iodine balance include analysis of intake, urine, stool and in some instances sweat. We used 3-day periods. We studied three subjects for five, one for three, and one for two consecutive periods. Our subjects included two normal persons, one patient with myxedema, and two patients with exophthalmic goiter. With the exception of subject E, no one had any iodine medication during the past 2 months.

The diet for each balance study was selected by the subject and remained unchanged throughout the experiment. The diets were calculated to contain 1 gm. of protein per kilo of body weight, and to have a caloric value of basal plus 20 to 30 per cent. The subjects were kept at bed rest and this regimen maintained them at constant weight.

An effort was made to secure as much uniformity as possible in the food used throughout the experiment. Canned goods for the entire study were of the same brand and bought at the same time. Meat and potatoes for the entire experiment were set aside at one time. Meat was ground, weighed, and frozen. Butter enough for the entire experiment was worked into one sample. Perishables were set aside for each period. Bread for 1 day was taken from a single loaf. The same brand was used throughout the experiments. Daily samples of the food except butter, sugar and salt, were weighed out and dried. Three-day samples were put together, ground, salt and sugar added, and aliquots taken for analysis. Butter and water were analyzed separately.

Daily urine samples were made up to 2 liters. Three-day samples were combined for analysis. Carmine was given to mark the feces 20 to 30 minutes before breakfast at the beginning of each period. The stools were divided, dried, and ground for analysis. When sweat was collected, the subjects wore loosely woven cotton underwear which had been washed in double distilled water. Towels which had been washed in double distilled water were used for bathing. Each day the underwear was changed. The subjects were bathed in 6 liters of water. The underwear and towels were washed through this water and then they were rinsed three times, making the total volume 10 liters. Aliquots from 3 days were combined for analysis. With C and D, tap water was used. For E, iodine-free water prepared by distillation from base was used.

The analyses on A and B were done by Phillips and Curtis' ('34) modification of the von Fellenberg method and those on C, D and E by McCullagh's method ('34).

The protocols of the subjects used in this experiment are given below:

Subject A, 336247, unemployed white male, 28 years of age entered the hospital 12/3/33 for this balance study. Physical and laboratory examinations were negative. Weight 120 pounds, B.M.R. minus 6, T. — 98, P. — 60, R. — 12, B.P. 92/60. He was placed on the diet 12/4/33 and the first carmine given 12/6/33.

Subject B, 340597, white female shoe stitcher, 31 years of age, entered the hospital 2/3/34 for this iodine balance study. Weight 109 pounds, B.M.R. minus 10, T. — 98.2, P. — 69, R. — 16, B.P. 110/70. The subject was placed on the diet 2/5/34, and the first carmine given 2/7/34.

Subject C, 335531, white housewife, 38 years of age, entered the hospital 10/27/33 with the signs and symptoms of essential hypertension. 11/31/33 thyroidectomy was performed leaving at the left a portion not larger than  $10 \times 7 \times 3$  mm., and on the right a portion not larger than  $10 \times 10 \times 2$  mm. B.M.R. before the operation was plus 3, and 2 weeks following operation it was minus 13. 4/8/34, 4½ months after thyroidectomy, the patient entered the hospital for a general check-up presenting a characteristic picture of myxedema. B.M.R. was minus 28, T. — 97, P. — 56, and the weight was 133, or 13 pounds higher than before operation. The subject was placed on the diet 4/10 and the collections started 4/12.

Subject D, 341454, white housewife, 24 years of age entered the hospital 3/27/34 with the following complaints of 5 months' duration: prominent eyes, nervousness, tremor, emotional and heat instability, tachycardia, dyspnea and hoarseness. Positive physical findings were a soft diffusely enlarged thyroid, pulse of 120, a soft systolic murmur best heard over the apex and transmitted over the entire precordium, blood pressure 148/84. 4/10/34 the patient was started on the experimental diet, and the first carmine was given 4/12. B.M.R. taken during the experiment was plus 28, T. — 98, P. — 100, R. — 20, B.P. 110/46.

Subject E, 345595, white housewife, 24 years of age, entered the hospital 10/30/34 with the following complaints of 6 months' duration: nervousness, ease of fatigue, enlargement of neck, and tachycardia. The patient had rubbed a patent medicine on her neck for 2 months and stopped using it 2 weeks before entering the hospital. This medicine was found to contain 1.3 gm. of iodine per 100 cc. We did not obtain this part of the history until the study was nearly finished. The patient had denied medication on three different occasions when the history was being checked. Positive physical findings were a warm moist skin, enlarged thyroid, pounding heart with shock over the apex, pulse 100, and fine tremor. The B.M.R. was plus 65, T. — 98.4, P. — 98, R. — 15, B.P. 120/60.

## RESULTS AND DISCUSSION

Subject A ingested 156 micrograms of iodine per day; the observations are shown in figures 1 and 2 as micrograms of iodine per day. The results on A were as follows: Period I, in the urine 51, in the stool 38, combined 89, positive balance 67; period II, in the urine 44, in the stool 19, combined 63, positive balance 93; period III, in the urine 53, in the stool 30, combined 83, positive balance 73; period IV, in the urine 43, in the stool 31, combined 74, positive balance 82; period V, in the urine 47, in the stool 16, combined 63, positive balance 93. The average urinary iodine output for the 15 days studied was 47, fecal iodine 27, combined 74, and balance 82. Only 47 per cent of the intake was accounted for by the urine and feces. This is less iodine in the urine and stool than Scheffer found in a subject of similar intake but the same as one with slightly lower intake.

Subject B ingested 56 micrograms of iodine per day and the results are shown in figures 3 and 4. The results on B were as follows: Period I, in the urine 36, in the stool 8, combined 44, positive balance 12; period II, in the urine 36, in the stool 6, combined 42, positive balance 14; period III, in the urine 36, in the stool 9, combined 45, positive balance 11; period IV, in the urine 31, in the stool 10, combined 41, positive balance 15; period V, in the urine 35, in the stool 12, combined 47, positive balance 9. The average urinary iodine output was 35 micrograms per day, fecal 9, making a combined excretion of 44 micrograms of iodine per day or 75 per cent of the iodine intake. Von Fellenberg accounted for about the same percentage on himself when he ingested 17 micrograms of iodine per day and also 80 micrograms. One of Scheffers' subjects on a higher intake showed a similar percentage output. The other subjects tended to agree more nearly with the results on A.

Sweat loss may have been an important factor in the great difference between A and B. A had a consistently moist skin and was restless, while B had a dry skin and remained quietly in bed. The great importance of sweat had been emphasized



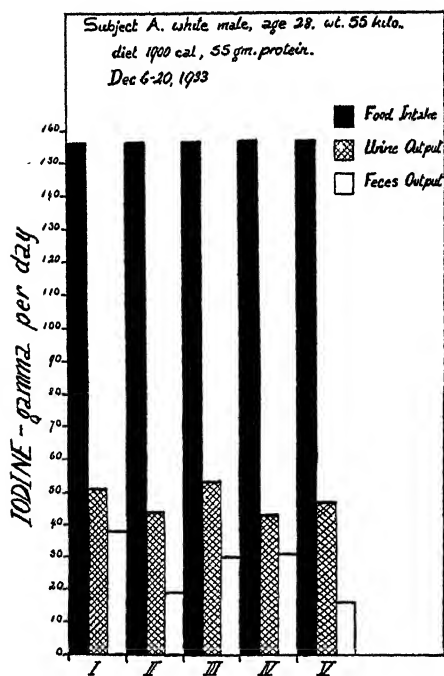


Figure 1

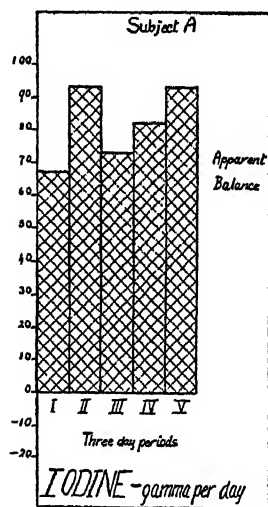
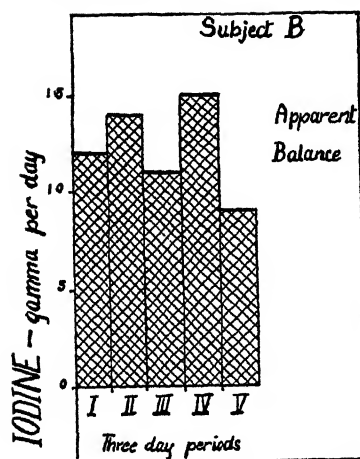
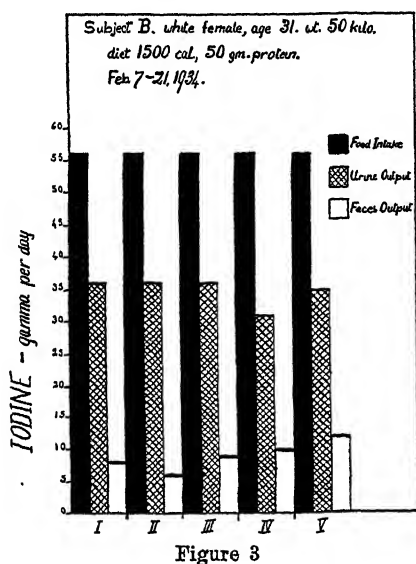


Figure 2

by Scheffer but we had hoped by bed rest in a room of 70 to 75°F. to render this factor constant. The effect of a previous diet, shown by von Fellenberg's work, may have been a con-



tributing factor here. However, the relative constancy on five consecutive periods leads us to believe that this is not very probable.

In the next three subjects, on which we made sweat determinations, balances are given as apparent balances without the sweat being subtracted, and as balance when the sweat is considered. The results on C are shown in figure 5. Two periods only were run. The amount of iodine ingested was 39 micrograms per day. The output was as follows: Period I, in the urine 24, in the stool 9, through the skin 19 micrograms per day; period II, in the urine 20, in the stool 10, and through the skin 20 micrograms per day. This gives a positive balance of

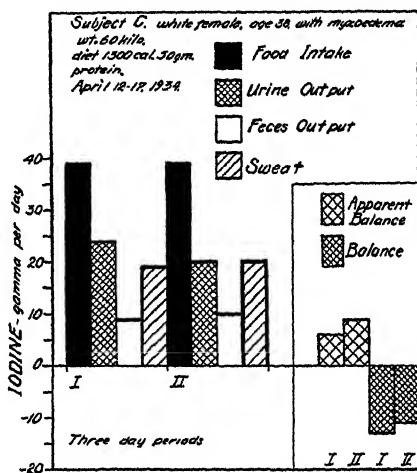


Figure 5

6 and 9 micrograms per day, not considering the sweat excretion or a negative balance of 13 and 11 when the sweat is considered. This experiment was not continued long enough to draw any conclusions unless many more such studies show similar results. We are at a complete loss to explain the great amount of iodine excreted through the skin in a case of true myxedema.

The results on D are shown in figures 6 and 7. The intake of iodine was 162 in periods I, II, III, and 147 in periods IV and V. Some vegetables were changed at the request of the patient in periods IV and V. The output was as follows: Period I, in the urine 46, in the stool 94, in the sweat 15, total

155, positive balance 7; period II, in the urine 63, in the stool 90, in the sweat 16, combined 169, negative balance 7; period III, in the urine 43, in the stool 99, in the sweat 13, total 155, positive balance 7; period IV, in the urine 64, in the stool 95,

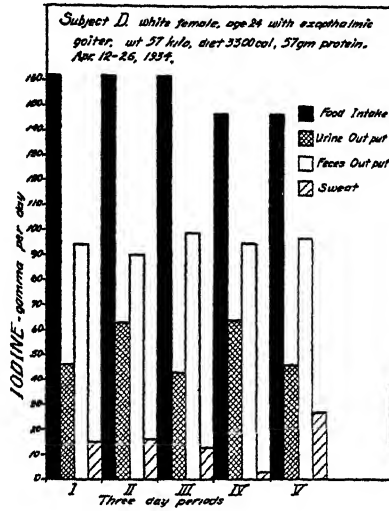


Figure 6

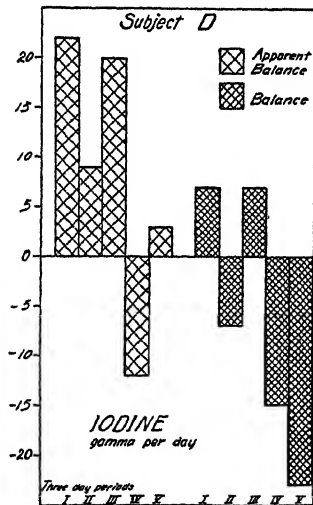


Figure 7

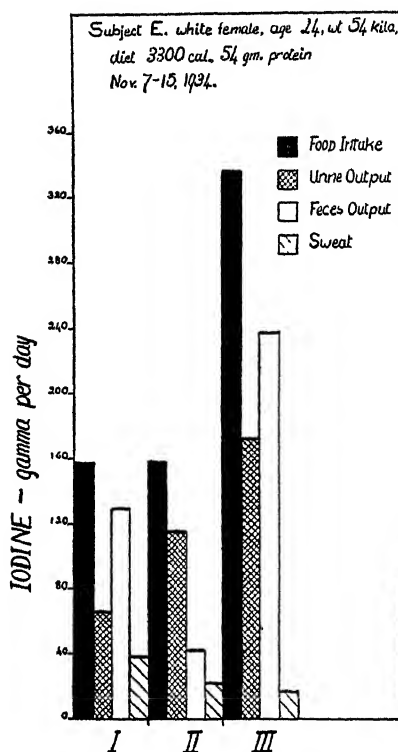


Figure 8

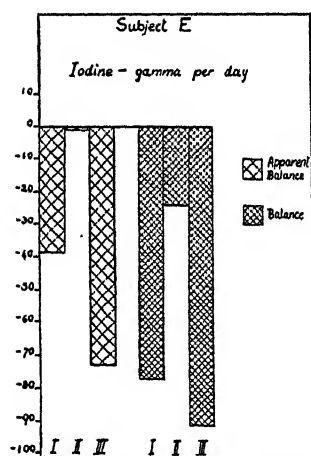


Figure 9

in the sweat 3, total 162, negative balance 15; period V in the urine 46, in the stool 97, in the sweat 27, combined 170, negative balance 24. It will be noticed that the striking thing here is the large output of iodine in the feces.

The results on subject E are shown in figures 8 and 9. The iodine ingested in the food was 158 micrograms per day throughout, but in the third period the iodine intake was raised to 336 with potassium iodide. The iodine excretion was as follows: Period I, in the urine 67, in the stool 130, in the sweat 38, combined 235, negative balance 77; period II, in the urine 116, in the stool 43, in the sweat 23, combined 182, negative balance 24; period III, in the urine 172, in the stool 237, in the sweat 18, combined 427, negative balance 91. The large negative balance here is perhaps a result of the iodine medication received 2 weeks previously. We may assume, however, that since the fecal iodine is little influenced by small changes in intake previous medication would have little effect on it. Therefore this would appear to be further evidence for Scheffer's contention that there is an increased fecal loss of iodine in hyperthyroidism. The reason for our not confirming the very large sweat loss in hyperthyroidism found by Scheffer is doubtless due to the fact that our patients were kept comfortable because it was easier to handle them that way. This meant that their rooms were a little cooler and they had less bedding than the other subjects.

#### SUMMARY

1. Iodine balance was studied in 3-day periods for 15 days on three subjects, nine on a fourth, and six on a fifth.
2. The combined urine and stool iodine may account for less than 50 per cent of the iodine intake.
3. There was a fairly uniform output of iodine for an individual on a monotonous diet.
4. The fecal iodine was higher in these studies than in those of Scheffer and von Fellenberg.
5. In two subjects studied Scheffer's observation of an increased fecal iodine in hyperthyroidism was confirmed.

## ACKNOWLEDGMENT

The authors wish to acknowledge the invaluable cooperation of Mary L. Bone, dietician of the Starling-Loving Hospital, and Ione Sundstrom, head nurse of the Research Division of the hospital.

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## CORRECTION

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Page 513, line 18. Sentence beginning "When half of the inulin of diet 11" should read "When the amount of inulin present in diet 11 was added as a supplement to the basal sucrose-containing diet (diet 10 a) all the animals grew well, an average gain per rat of 36.3 gm. being recorded."





# THE UTILIZATION OF INULIN FOR GROWTH BY THE YOUNG WHITE RAT

ADELAIDA BENDAÑA AND HOWARD B. LEWIS

*Department of Biological Chemistry, Medical School, University of Michigan,  
Ann Arbor*

ONE FIGURE

(Received for publication August 5, 1935)

Despite much experimental study, the value of inulin for the nutrition of the higher forms is yet to be demonstrated satisfactorily. It is recognized that enzymes, which convert inulin to fructose, can hardly be significant factors in the activity of the secretions of the gastro-intestinal canal of the higher forms, but that under certain conditions, the gastric acidity may be sufficiently high to cause the hydrolysis of inulin to fructose to a limited extent. The microflora of the intestine attack inulin readily, but the chief products of this fermentation are fatty acids of low molecular weight and not carbohydrates (Ashe and Bing, '34). Absorption and utilization of inulin without previous hydrolysis in the gastro-intestinal tract has also been suggested (Heupke and Blanckenburg, '34), but in the light of experiments on the fate of inulin introduced parenterally, this seems improbable. McCance and Lawrence ('29) have presented an excellent critical review of the general problem of inulin utilization.

The chief evidence in support of utilization of inulin is to be obtained from certain experiments in which a deposition of liver glycogen has been observed to follow inulin feeding and from increases in respiratory quotients after the administration of inulin. In most cases, the increases in liver glycogen after ingestion of inulin are slight, particularly

when compared with the values obtained after the feeding of comparable amounts of readily utilized carbohydrates, fructose or glucose. The fallacies of the evidence based on the respiratory quotient are well presented by McCance and Lawrence ('29).

In addition to lack of satisfactory direct experimental methods and to difficulties in the interpretation of data, still further confusion has resulted from the failure to recognize the variability of the material used as a source of the carbohydrate. It is known that the chemical and physical properties of inulins of different botanical origin vary (Yanovsky and Kingsbury, '31, '33), and that fructosans, other than inulin, are present in plant tissue. Moreover the relative amounts of inulin and the other fructosans, the so-called inulides, vary with the season. It can hardly be expected, therefore, that the results obtained by the administration of a tuber (e.g., Jerusalem artichoke), which contains inulin and other fructosans, will be comparable with those obtained with inulin isolated from the same source. In fact inulins of different origin, although prepared under uniform conditions, may not be chemical individuals, and it is preferable in the light of our present knowledge to include in the inulin group all complex anhydrides of fructose (Yanovsky and Kingsbury, '31).

One direct method of study, which is concerned with the calorific value of inulin in the diet of the young white rat, has not been employed extensively. If inulin is used to any significant extent, it should be able to supply energy and thus promote the growth of young white rats when supplied either as the sole source of carbohydrate, or as a supplement to readily available carbohydrates in a low calorie diet adequate in all other known essential elements. The present study employs this procedure and approaches the inulin problem by a consideration of the rate of growth of young white rats maintained on a low calorie diet with sucrose or fructose as the source of carbohydrate in the basal diet and inulin or inulin plus sucrose or fructose in the experimental diets.

Purified inulin from chicory, generously furnished through the courtesy of Dr. Elias Yanovsky of the carbohydrate division of the Bureau of Chemistry and Soils of the United States Department of Agriculture, was used in our studies.

The results have indicated some utilization of inulin as a source of energy for the growing white rat, a utilization, however, distinctly inferior to that of either sucrose or fructose. In common with other observers, we have noted that inulin, if ingested in considerable amounts, resulted in marked gas formation and disturbance of function of the gastro-intestinal canal. We feel that our data indicate that the isolated polysaccharide from chicory is of little dietetic value from the practical standpoint as a significant source of energy. Our results are at variance with those of certain Japanese investigators, which will be considered subsequently.

#### EXPERIMENTAL

The chicory inulin used in these studies contained 10 per cent of moisture and 0.09 per cent of ash and showed a specific rotation of  $-33.9^{\circ}$  (uncorrected for moisture or ash) when examined at  $27^{\circ}$  with sodium light.

Litter units of young white rats of 65 to 75 gm. weight were divided into control and experimental groups for the nutritional studies to be described. Attempts to use younger and smaller rats were unsuccessful since, even when relatively small amounts of inulin (1.5 gm.) were added as supplements to the basal diet, marked diarrhoea and distention of the intestines resulted and the animals died within a few days.

In preliminary experiments it was found that inulin was not satisfactory as the sole source of carbohydrate of the diet, that some readily utilizable carbohydrate must be supplied. The addition of 2.5 to 4.1 gm. of inulin to a basal diet containing no readily available sugar resulted in a rapid loss of weight and death within a few days. Death was presumably due to starvation and the marked distention of the intestines occasioned by the large amount of gas produced as a result of the fermentation of the inulin. The distention of

the abdomen was so marked that the animals could be readily distinguished from the controls. Diarrhoea and frequently bloody fecal masses were observed.

In the final series of experiments, basal low calorie diets containing a limited amount of sucrose (diet 10) or fructose (diet 12) were prepared to which extra carbohydrate in the form of sucrose, fructose or inulin was added for the experimental diets. The composition of these diets is presented in table 1.

TABLE 1  
*Basal and experimental diets*

	10	10a	10c	11	12	12a	12c
	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Casein	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Cod liver oil	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Lard	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Agar	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Salt mixture <sup>1</sup>	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Sucrose	1.5	1.5	3.0				
Inulin		1.5		1.5		1.5	
Fructose					1.5	1.5	3.0
Daily food intake	3.8	5.3	5.3	3.8	3.8	5.3	5.3
Daily calorie intake (calculated)	12.6	18.6 <sup>2</sup>	18.6	12.6 <sup>2</sup>	12.6	18.6 <sup>2</sup>	18.6

<sup>1</sup> McCollum-Davis salt mixture no. 135.

<sup>2</sup> Calculated on the basis that 1 gm. of inulin supplies the same number of calories as 1 gm. sucrose or fructose.

In each series of experiments, the rats were placed in individual wire cages and given a fixed amount of food daily, 3.8 gm. for each of the animals receiving diets 10, 11 and 12, and 5.3 gm. for each of those receiving diets 10a, 12a, 12c and 10c. A supplement of 50 mg. of Harris yeast vitamin pill was given daily to each rat to supply the vitamin B complex. In two series of experiments a daily supplement of 100 mg. of the vitamin pill was given daily to each rat to see whether a larger amount of vitamin B would influence the growth of the rats. Water was given ad libitum. The rats were weighed every 3 days. Consumption of food was both rapid and complete. This was to be expected as the amount of food given daily to each rat was quite small (3.8 or 5.3 gm.).

Typical results obtained with various litter units are presented in table 2 and figure 1. The diets fed were essentially the same except for the content of carbohydrates. The basal diet, which furnished approximately 12.6 Cal., was calculated to be sufficient to maintain the animals at nearly constant

TABLE 2

*Weight of rats fed low calorie diets and diets containing added carbohydrate. All feeding periods were of 60 days duration*

SEX	DIET	WEIGHT		CHANGE IN WEIGHT	SEX	DIET	WEIGHT		CHANGE IN WEIGHT
		Initial	Final				Initial	Final	
Litter A					Litter B				
		gm.	gm.	gm.			gm.	gm.	gm.
•F	10	60.6	65.5	+ 4.9	F	12	70.0	75.0	+ 5.0
F	10	71.5	81.5	+ 10.0	M	12	70.2	75.5	+ 5.3
M	10	65.0	80.0	+ 14.5	M	12	64.5	81.0	+ 16.5
F	11	69.0	62.5	— 6.5	F	12	64.5	78.5	+ 14.0
M	11	76.0	62.0	— 14.0	M	12c	77.5	113.5	+ 36.0
F	11	76.0	57.5	— 18.5	F	12c	62.5	102.5	+ 40.0
F	10a	70.5	102.0	+ 31.5	F	12c	71.5	112.0	+ 40.5
M	10a	64.5	105.0	+ 40.5	M	12c	70.5	119.0	+ 49.0
F	10a	67.0	99.0	+ 32.0	F	12a	70.1	99.0	+ 28.9
F	10a	63.1	104.5	+ 41.4	F	12a	67.5	99.5	+ 32.0
Litter C					M	12a	67.0	94.5	+ 27.0
F	10	71.0	74.0	+ 3.0	Litter D				
F	10	63.5	68.0	+ 4.5	M	12	60.8	67.5	+ 6.7
M	10	68.5	79.5	+ 11.5	F	12	65.2	68.5	+ 3.3
F	10a	77.0	98.5	+ 21.5	F	12	59.0	68.5	+ 9.5
F	10a	71.0	86.5	+ 15.5	M	12	52.0	60.5	+ 8.5
M	10c	82.5	119.8	+ 37.3	F	12a	61.0	81.5	+ 20.5
F	10c	72.0	113.5	+ 41.5	M	12a	69.0	87.0	+ 18.0
F	10c	74.2	107.3	+ 33.1	M	12	73.2	87.5	+ 14.3
					F	12	58.5	79.5	+ 21.0

weight. If the inulin were utilized to any significant extent, addition of 1.5 gm. of inulin daily (6 Cal.) should result in increased growth, since it was considered that the protein of the diet was adequate for animals of this weight. In the first series of experiments, in which sucrose served as the source of carbohydrate in the basal diet (diet 10), added inulin

(diet 10a) produced definite increases in weight (table 2, litters A and C), indicating utilization of the polysaccharide. Since inulin is a fructosan, it was considered desirable to supply fructose rather than sucrose as the source of carbohydrate in the basal diet (diet 12). Addition of inulin to this diet (diet 12a) also resulted in an increased rate of growth (table 2, litters B and D). It appeared that inulin was available as a source of energy to some extent.

In order to determine how the utilization of inulin compared with that of sucrose or fructose under our experimental

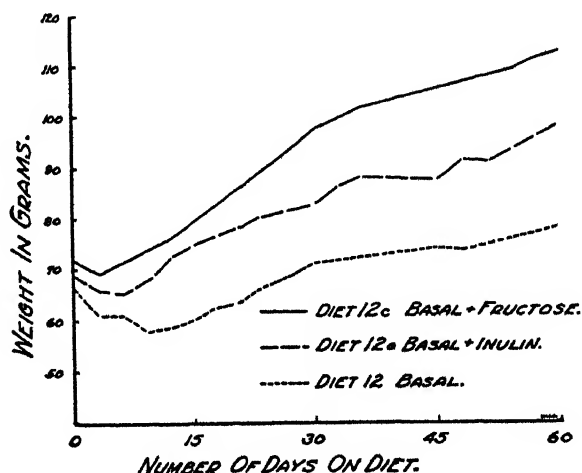


Fig. 1 Weight curves of rats receiving the basal low calorie diet and the basal diet supplemented by inulin and by fructose. The curves presented are averaged for three (diet 12a) and four (diets 12 and 12c) rats of the same litter.

conditions, sucrose (diet 10a) and fructose (diet 12c) were added to the basal diet in amounts comparable to the amount of the inulin added. It was observed that while, as already stated, rats fed the basal diets plus 1.5 gm. of inulin showed better growth than the control animals receiving the basal diet without supplement, the animals receiving the basal diet plus sucrose (litter C) or fructose (litter B) showed rates of growth distinctly superior to that of the inulin-fed rats. The figures show that although inulin may be utilized by the rat, the extent of utilization as measured by the growth response

is by no means comparable to that of either sucrose or fructose. The same conclusion is to be drawn from the typical growth curve presented in figure 1.

It has been stated previously that it was found necessary to include some readily utilizable carbohydrate in the diet and that when inulin was the sole source of carbohydrate, marked gastro-intestinal disturbances which terminated fatally within a few days resulted. With one litter only were we successful in continuing the use of the diet in which inulin was the sole source of the carbohydrate (diet 11) throughout the entire feeding period of 60 days. The results of this experiment are presented in table 2 (litter A). On the basal low calorie diet containing a limited amount of sucrose (diet 10), three animals gained an average of 9.8 gm. each in the 60-day period. When inulin served as the sole source of carbohydrate (diet 11), all the animals survived but lost weight at first rapidly, then more slowly (an average loss of weight of 13.0 gm.). When half of the inulin of diet 11 was replaced by sucrose (diet 10a), all the animals grew well, an average gain per rat of 36.3 gm. being recorded. The results with this litter are discussed in detail since these particular animals appeared to tolerate inulin unusually well and the group fed both sucrose and inulin showed growth superior to that obtained on inulin feeding with any other litter. These results show clearly that inulin alone as a source of carbohydrate is unsatisfactory, but that inulin as a supplement to a diet containing some other carbohydrate has a limited value.

Our results which indicate that the utilization of inulin as a source of energy by young white rats is limited and is distinctly inferior to that of sucrose or fructose are not in accord with the results of a few similar studies in the literature. Emerique ('25) in studies similar to those here reported was unable to observe any utilization of inulin by young white mice. One group of only three animals was studied however. Ariyama and Takahasi ('29) believed that the nutritive value of inulin was equal to that of starch, dextrin, sucrose or lactose. The small number of rats (two) used makes the



experimental results difficult to interpret. Narushima ('27) found that inulin was as effectively utilized by young white rats as was cornstarch, but was not as effectively utilized as fructose. It seems incredible that the addition of 0.8, 2.4 and 2.8 Cal. as fructose (i.e., 0.2, 0.6 and 0.7 gm.) to a basal diet furnishing 37 Cal. daily could lead to such marked increases in weight (37.5, 37.5 and 34.5 gm., respectively), while the animals on the basal diet gained only 8.5 gm. during the same experimental period of 33 days. These results are the more striking since these small variations in the calorific value of the diet markedly influenced the growth of animals weighing for the most part over 100 gm. at the beginning of the feeding periods. Our results are rather in harmony with previous work from this laboratory (Bodey, Lewis and Huber, '27) in which it was shown that the administration of inulin led to an increased storage of glycogen in fasting young white rats but that the storage of glycogen was much less marked than that observed when fructose was fed.

In conclusion we wish to emphasize that, while our evidence indicates that the organism of the young rat may utilize inulin as a source of energy to a limited degree, the experiments furnish no information as to how this energy is made available. The lack of a known effective mechanism for the hydrolysis of inulin to fructose in the gastro-intestinal tract of the higher forms and the marked intestinal fermentation observed suggest that the nutritive value of the inulin may be due to the absorption and utilization of organic acid or similar products formed in the intestine by the activities of microorganisms (Ashe and Bing, '34). In addition, it should be pointed out that the results of the investigation do not bear directly on the problem of the utilization of inulin-containing vegetables. Our investigations are concerned with the isolated polysaccharide, inulin, from a single botanical source, chicory,<sup>1</sup> and the results cannot be applied to the problem of

<sup>1</sup>We also prepared in this laboratory inulin from freshly dug dahlia tubers. A limited series of studies with dahlia inulin gave results comparable with those obtained with inulin from chicory.

the utilization of the mixture of fructosans, the inulides, present in the vegetables, the nature of which varies with the season and with the botanical species.

#### SUMMARY

1. Young white rats (65 to 75 gm.) were maintained upon a diet, whose limiting factor was a calorific one.

2. When extra calories in the form of inulin from chicory, sucrose, or fructose were supplied as a supplement to the basal diet, the rate of growth was increased. The utilization of inulin under these experimental conditions, as measured by the response in growth, was distinctly inferior to that of sucrose or fructose.

3. Inulin could not be effectively utilized by the young white rat as the sole source of carbohydrate since the marked intestinal fermentation of the polysaccharide caused distention of the intestine and usually death within a few days.

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# THE EFFECT OF CEREAL DIETS ON THE COMPOSITION OF THE BODY FAT OF THE RAT<sup>1</sup>

HAROLD S. OLCOTT,<sup>2</sup> WILLIAM E. ANDERSON AND  
LAFAYETTE B. MENDEL

*Department of Physiological Chemistry, Yale University, New Haven*

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Numerous studies have shown that the character of the body fat is materially modified by the diet. In feeding experiments with rats, Anderson and Mendel ('28) reported that with some dietary oils—soybean, corn, cottonseed and peanut—a close parallelism existed between the iodine number of the body fat and the iodine number of the food fat when the latter furnished 60 per cent of the total energy intake. Body fat synthesized from either carbohydrate or protein, so-called physiological fat, exhibited an iodine number of about 63. The present communication relates to a study of the character of fat produced when rations rich in cereals, which contain large amounts of carbohydrate and comparatively small amounts of fat, are fed. Do the individual cereal grains, corn, oats, wheat and barley, with an oil content ranging from 1.7 per cent to 5.3 per cent yield body fats of unlike composition? Inasmuch as cereal grains furnish the principal energy foods of human beings as well as of animals on which man depends for meat, milk, eggs, leather and wool, studies regarding any aspects of their nutritive properties ought to prove of interest and value.

<sup>1</sup> Presented before the Division of Biological Chemistry, Eighty-fourth meeting of the American Chemical Society, Denver, August 23, 1932.

<sup>2</sup> National research council fellow in medicine (1931-1932).

## EXPERIMENTAL

Yellow corn, oat groats, wheat and hulled barley were used in the experiments. The analyses of these grains and of dried skimmed milk are recorded in table 1. With the exception of the determination of the oil content of each grain, analyses of the grains and of dried skimmed milk were made according to conventional methods. The amount of each cereal oil was determined by continuous extraction with hot alcohol; the extract was evaporated in vacuo, and the residue extracted with ether; the ethereal solution was dried with anhydrous sodium sulfate, filtered, and evaporated. The oil thus obtained was dried to constant weight. Diets containing

TABLE 1  
*Analyses of cereals and dried skim milk*

CEREAL	MOISTURE	ASH	PROTEIN <sup>1</sup>	FAT	CARBOHYDRATE <sup>2</sup>
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Yellow corn	8.5	1.3	8.7	4.0	77.5
Oat groats	6.9	2.0	16.1	5.3	69.7
Wheat (mixed)	8.5	1.7	11.1	1.7	77.0
Hulled barley	9.1	1.6	9.9	2.0	77.4
Dried skim milk	4.0	8.2	36.3	0.8	50.7

<sup>1</sup> Cereals, N  $\times$  5.7; dried skim milk, N  $\times$  6.38.

<sup>2</sup> By difference.

80 per cent finely ground cereal, 18.5 per cent dried skim milk, and 1.5 per cent salt mixture, were fed. The salt mixture was devised to supplement the mineral elements of the other dietary constituents. The composition of the diets and the relationship of the energy value of each cereal oil to the total energy value of the respective diet are recorded in table 2.

Each diet, supplemented once weekly with a cod liver oil concentrate rich in vitamins A and D, was fed ad libitum to a group of four or five female albino rats, which at weaning (21 days of age), and prior to initiation of the feeding experiments, were fasted for 48 hours in order to deplete the fat depots. The individual body weights at weaning ranged

from 35 to 45 gm. The feeding tests were conducted until the animals attained between approximately 190 and 220 gm. body weight.

The growth rate of the animals on the oat and corn diets was slightly superior to that shown by the normal growth curves for female rats (Smith and Bing, '28). Regarding the wheat and barley fed groups, the rate of growth of the former group approximated the normal, whereas the animals raised on the barley ration grew below the normal rate; the

TABLE 2  
*Composition of diets*

DIETARY COMPONENT	OIL IN CEREAL	ENERGY VALUE OF CEREAL OIL	WEIGHT OF DIETARY COMPONENT	ENERGY VALUE OF DIETARY COMPONENT
	<i>per cent</i>	<i>per cent of total calories</i>	<i>per cent</i>	<i>per cent of total calories</i> <sup>1</sup>
Cereal				
Corn	4.0	8.0	80.0	82.0
Oats	5.3	10.0	80.0	82.0
Wheat	1.7	3.0	80.0	82.0
Barley	2.0	4.0	80.0	82.0
Dried skim milk			18.5	18.0
Salt mixture <sup>2</sup>			1.5	

A dose of 20 mg. cod liver oil concentrate<sup>3</sup> was administered once weekly to each rat

<sup>1</sup> Approximate values. The values for four cereals ranged from 81.7 to 82.9 cal.

<sup>2</sup> The salt mixture consisted of calcium carbonate 55 per cent, sodium chloride 40 per cent, ferric citrate 4.5 per cent and potassium iodide 0.5 per cent.

<sup>3</sup> The concentrate was kindly furnished by the Health Products Corporation, Newark, N. J.

individuals of the last-mentioned group weighed 20 to 30 gm. less at 125 days of age than those of the other groups.

Each rat was killed with illuminating gas, the gastrointestinal tract removed, and the remaining tissue comminuted in a meat grinder. The lipids from this hashed tissue were obtained in most cases by a steam-rendering process described by Anderson and Mendel ('28). The lipids from the hashed tissue of one individual from each group were obtained by Bloor's ('26) hot alcohol-ether extraction method

and after the removal of acetone-insoluble substances (phospholipids), were compared in iodine value (table 3) with those obtained by the steam-rendering process which contained no acetone-insoluble substances. The iodine values were determined in duplicate by the Rosenmund and Kuhnhehn method

TABLE 3  
*Iodine number of body fats and of cereal oils*

TYPE OF DIET	FINAL BODY WEIGHT OF RATS	IODINE NUMBER OF BODY FATS <sup>1</sup>	IODINE NUMBER OF CEREAL OIL <sup>1</sup>
Corn	<i>gm.</i>		
	219	81	116
	220	83	
	220	83	
	220	83	
	219	86 <sup>2</sup>	
		Average 83	
Oats	220	76	99
	220	78	
	220	79	
	220	82 <sup>2</sup>	
	220	82	
		Average 79	
Wheat	219	66	110
	176	70 <sup>2</sup>	
	220	71	
	219	72	
		Average 70	
Barley	198	68	101
	188	69	
	188	71 <sup>2</sup>	
	195	72	
	191	73	
		Average 71	
'Fat-free'		68 <sup>2</sup>	(Eckstein, '29)
		64-71 <sup>2</sup>	(McAmis, Anderson and Mendel, '28-'29).

<sup>1</sup> Rosenmund-Kuhnhehn method.

<sup>2</sup> Extracted with hot alcohol.

<sup>3</sup> Hanus method.

as modified by Yasuda ('31). In order to obtain further knowledge in regard to the make-up of the body fat, a sufficiently large sample, obtained by pooling all individual samples of each experimental group, was separated into its component saturated and unsaturated fatty acids by the lead-salt-ether method as described by Jamieson ('32). The data obtained are recorded in table 4.

TABLE 4  
*Distribution of fatty acids in body fat of rats<sup>1</sup>*

TYPE OF DIET	IODINE NUMBER OF BODY FAT	TOTAL FATTY ACIDS RECOVERED	SATURATED FATTY ACIDS	UNSATURATED FATTY ACIDS	IODINE NUMBER OF UNSATURATED FATTY ACIDS
		<i>per cent</i>	<i>per cent<sup>2</sup></i>	<i>per cent<sup>2</sup></i>	
Corn	83	90.4	24.6	75.4	114
		90.0	25.3	74.7	116
Oats	79	93.1	28.5	71.5	114
		91.0	29.3	70.7	114
Wheat	70	93.3	30.0	70.0	103
		91.8	30.5	69.5	105
Barley	71	92.9	31.6	68.4	108
		93.2	31.2	68.8	108

<sup>1</sup> The average of two iodine number determinations of body fat is recorded; other figures represent the result of an individual analysis.

<sup>2</sup> Corrected percentage. The iodine numbers of the saturated acid fractions ranged from 6 to 11.

#### DISCUSSION

The average iodine numbers of 70 and 71 for the body fat, respectively, produced by rats fed the wheat and barley diets indicate that these animals deposited fat resembling that produced on 'fat-free' diets (Eckstein, '29; McAmis, Anderson and Mendel, '28-'29), whereas those fed corn and oats deposited fat definitely more unsaturated (table 3). Despite the fact that the oil of oats was less unsaturated (I.N. 99) than any of the other cereal oils, the comparatively large oil content (5.3 per cent) is presumably responsible for the unsaturation (I.N. 79) of the body fat of rats fed the oats ration. Corn



oil was more unsaturated (I.N. 116) than any of the other oils, and the body fat of the rats fed the diet containing corn was correspondingly more unsaturated (I.N. 83) than the body fats of the other groups.

A study of the fatty acid composition of the various types of body fat indicated that the differences in iodine numbers were dependent both on the amount and character of the unsaturated fatty acids present (table 4). The body fat of the rats fed the corn diet contained the highest percentage of unsaturated acids and this fraction was the most unsaturated (I.N. 115) obtained. The body fat of the other three groups contained approximately the same percentage of unsaturated acids but differed in the iodine numbers of this fraction. The results suggest that some of the food unsaturated fatty acids are stored in preference to 'synthetic' fat when the food fat exceeds a certain minimal level, which level might be dependent upon the degree of unsaturation of the dietary fat.

#### SUMMARY

The cereal grains, corn, oats, wheat and barley, each of which has a low oil content, were included in individual diets of rats to furnish 82 per cent of the total energy value of the ration. The oil of corn and oats, constituting 4.0 per cent and 5.3 per cent, respectively, of the grains, supplied 8.0 and 10.0 per cent of the total calories of the corn and oats rations, respectively. Body fat produced by corn- and oats-fed rats, differed slightly in degree of unsaturation as measured by the iodine number (83 and 79, respectively). The oil of wheat and barley constituting 1.7 per cent and 2.0 per cent of the respective grains, supplied only 3.0 and 4.0 per cent of the total energy value of the respective wheat and barley diets. Body fat yielded by animals fed wheat and barley rations was less unsaturated (I.N. 70 and 71, respectively) than fat produced on either corn or oats diets, and was similar in character to fat obtained from rats fed 'fat-free' diets. As a result of a study of the total saturated and total unsaturated fatty acids of body fat obtained from rats on each cereal

ration, the unsaturated fatty acid content was found to range from about 68.5 per cent for fat from those fed the barley diet to 75.0 per cent for fat of rats on the corn ration.

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# THE INFLUENCE OF DIET ON THE GLUCOSE TOLERANCE OF THE DOG

ESTHER M. GREISHEIMER AND F. W. HOFFBAUER

*Department of Physiology, University of Minnesota, Minneapolis*

## THREE FIGURES

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The preceding diet may influence the glucose tolerance markedly. This question has been studied by Greenwald ('24), du Vigneaud ('25), Malmros ('28), Tolstoi ('29), Heinbecker ('28), Sweeny ('27 and '28), and Himsworth ('33). The procedure varied with respect to the diet, the duration of the experimental feeding, and the subject used. These variations probably account for the differences observed and for the differences in opinion held by the various investigators.

## METHOD

The problem was to compare the effects of the different dietary regimes on the carbohydrate tolerance of each of six dogs. Three male and three non-pregnant female adult dogs were used. One male and one female dog of the original group become ill, and were replaced by two other dogs. The weight range was from 13 to 20 kg. The animals were mongrels, chosen from the supply of stock animals available at the time. The dogs were vermifuged before the experiment began. Each dog was kept in a large individual cage, and was allowed 6 hours of freedom daily in favorable weather, except on the days when glucose tolerance tests were performed.

The dogs were fed weighed portions of food twice each day, receiving one-half of their daily allowance at 9.00 A.M. and the other half at 3.00 P.M. Fresh water was available to each at all times except during the course of a glucose tolerance test. A fasting period of 16 hours preceded the glucose tolerance tests.

In selecting the diets, certain common foodstuffs were chosen, which could be combined in various ratios, to give the diets suited to the experimental needs. The composition of all the foods used in the experiment was taken from the values published by Atwater and Bryant ('06). The requirements to be satisfied were that the dogs would eat all the food given them, and neither gain nor lose weight to any extent. A value of 32 Cal. per kilogram per 24 hours was chosen as approximately the basal requirement for caged dogs, based on average figures reported by Steinhaus ('28). Each dog was given twice his basal requirement, or 64 Cal. per kilogram per 24 hours. This figure agrees fairly well with that of 70 Cal. per kilogram per day used by Morgulis and Edwards ('24) as a maintenance allowance for dogs under laboratory conditions.

The compositions of the various diets are shown below.

*Balanced diet.* Consisted of oatmeal and cornmeal (boiled together), boiled potatoes, lean steak, ground bacon, butter, and sodium chloride; 12.1 per cent protein, 27.8 per cent fat, and 60.1 per cent carbohydrate.

*High protein no. 1.* Consisted of granular gelatin, lean steak, cornmeal, ground bacon, and sodium chloride; 49 per cent protein, 19.93 per cent fat, and 31.07 per cent carbohydrate.

*High fat no. 1.* Consisted of boiled potatoes, lean steak, ground bacon, butter, and sodium chloride; 12.25 per cent protein, 75.24 per cent fat, and 12.51 per cent carbohydrate.

*High carbohydrate.* Consisted of boiled potatoes, cornmeal, honey, and sodium chloride; 8.82 per cent protein, 3.41 per cent fat, and 87.77 per cent carbohydrate.

*High protein no. 2.* Consisted of boiled egg, lean steak, cornmeal, bacon, and sodium chloride; 51 per cent protein, 22.6 per cent fat, and 26.4 per cent carbohydrate.

*High protein no. 3.* Consisted of boiled egg, lean steak, cornmeal, and sodium chloride; 74.4 per cent protein, 19.7 per cent fat, and 5.9 per cent carbohydrate.

*High protein no. 4.* Consisted of boiled egg, lean steak, cornmeal, bacon, and sodium chloride; 24.2 per cent protein, and 21.0 per cent fat, and 54.8 per cent carbohydrate.

*High fat no. 2.* Consisted of boiled egg, cornmeal, lean steak, bacon, butter, and sodium chloride; 15.9 per cent protein, 55 per cent fat, and 29.1 per cent carbohydrate.

*High fat no. 3.* Consisted of boiled egg, cornmeal, lean steak, bacon, butter, and sodium chloride; 13.8 per cent protein, 80.1 per cent fat, and 6.1 per cent carbohydrate.

In the first experimental period, the following scheme was used:

7 days on balanced; preliminary; dogs A to F, inclusive  
7 days on high protein no. 1 (using gelatin), ended 7/27/34  
7 days on balanced; ended 8/3/34  
7 days on high fat no. 1; ended 8/10/34  
7 days on balanced; ended 8/17/34  
7 days on high carbohydrate; ended 8/24/34  
7 days on balanced; ended 8/31/34

In the second experimental period following an interim of 2½ months, during which the animals were maintained on the regular stock diet, the following scheme was used:

7 days on balanced; dog G replaced A; ended 11/7/34  
7 days on high protein no. 2; ended 11/14/34  
7 days on high protein no. 3; ended 11/21/34  
7 days on high protein no. 2; ended 11/28/34; dog B became ill  
7 days on high protein no. 4; ended 12/5/34  
7 days on balanced; ended 12/12/34  
7 days on high fat no. 2; dog H replaced B; ended 12/19/34  
7 days on high fat no. 3; ended 12/26/34  
7 days on high fat no. 2; ended 1/2/35  
7 days on balanced; ended 1/9/35

Blood was drawn from the external jugular vein. Three grams of glucose per kilogram of body weight in 100 cc. of water were given by stomach tube; blood samples were drawn  $\frac{1}{2}$ , 1, 2 and 3 hours after the glucose was given.

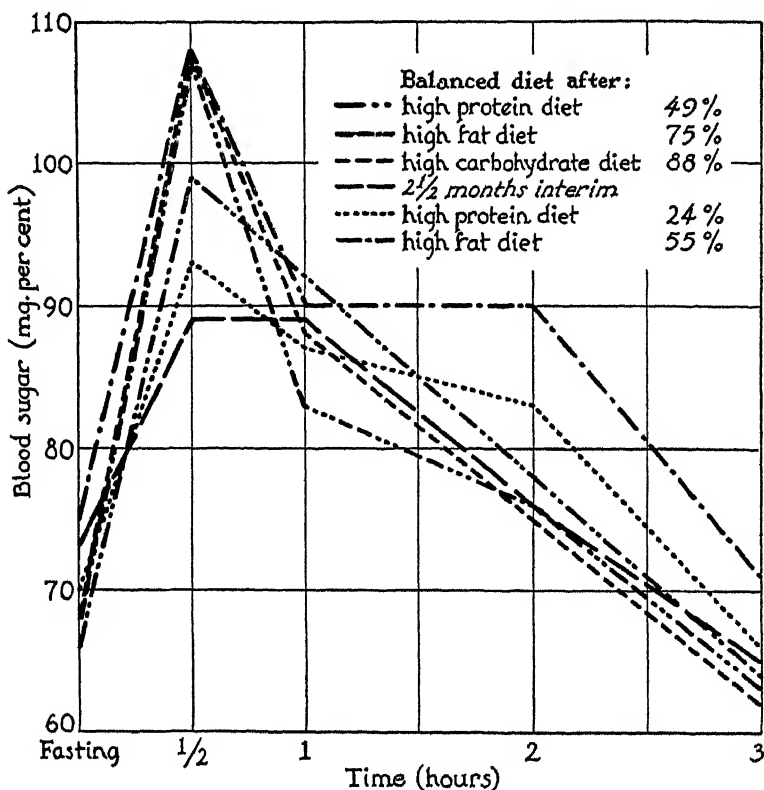


Fig. 1 Showing the residual effects of 24 and 49 per cent protein, 88 per cent carbohydrate, and 55 and 75 per cent fat diets; these are found after 7 days on the balanced diet, following the experimental diet.

Blood sugar was determined by the Shaffer-Somogyi ('33) method. All analyses were made in duplicate, using 1 cc. of blood for each. The average for the two determinations is used in the report. To prevent coagulation, potassium oxalate was used (2 drops of 20 per cent solution for each 3 cc. of blood). The blood was analyzed as soon as drawn, to prevent glycolysis.

## RESULTS

In the six periods of balanced diet, 35 glucose tolerance tests were performed. The results are shown below.

	<i>Fasting</i>	<i>½ hour</i>	<i>1 hour</i>	<i>2 hours</i>	<i>3 hours</i>
18 tests on male dogs	71	91	84	75	64
17 tests on female dogs	69	108	93	83	65
Average of 35	70	99	88	79	65

It will undoubtedly be more instructive to report the results on each period of balanced diet separately, to show

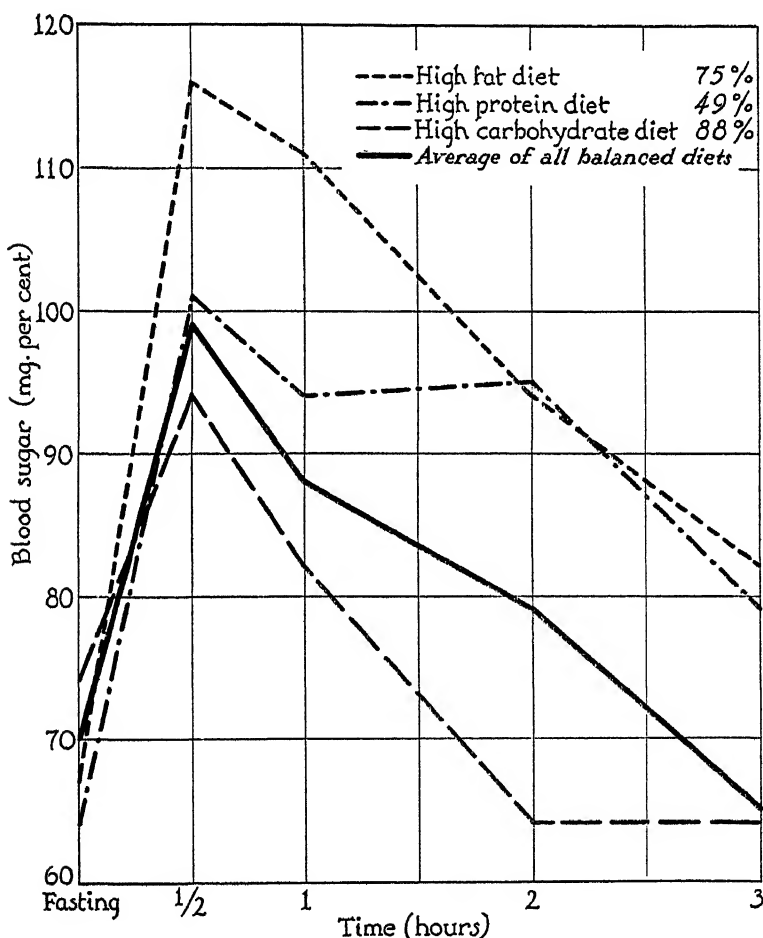


Fig. 2 Showing the effects of 75 per cent fat, 49 protein, and 88 per cent carbohydrate diets on the glucose tolerance of six dogs. First experimental period.



whether or not the effects of the experimental diets are still to be noticed, even after 7 days on the balanced diet. The average results are shown in figure 1.

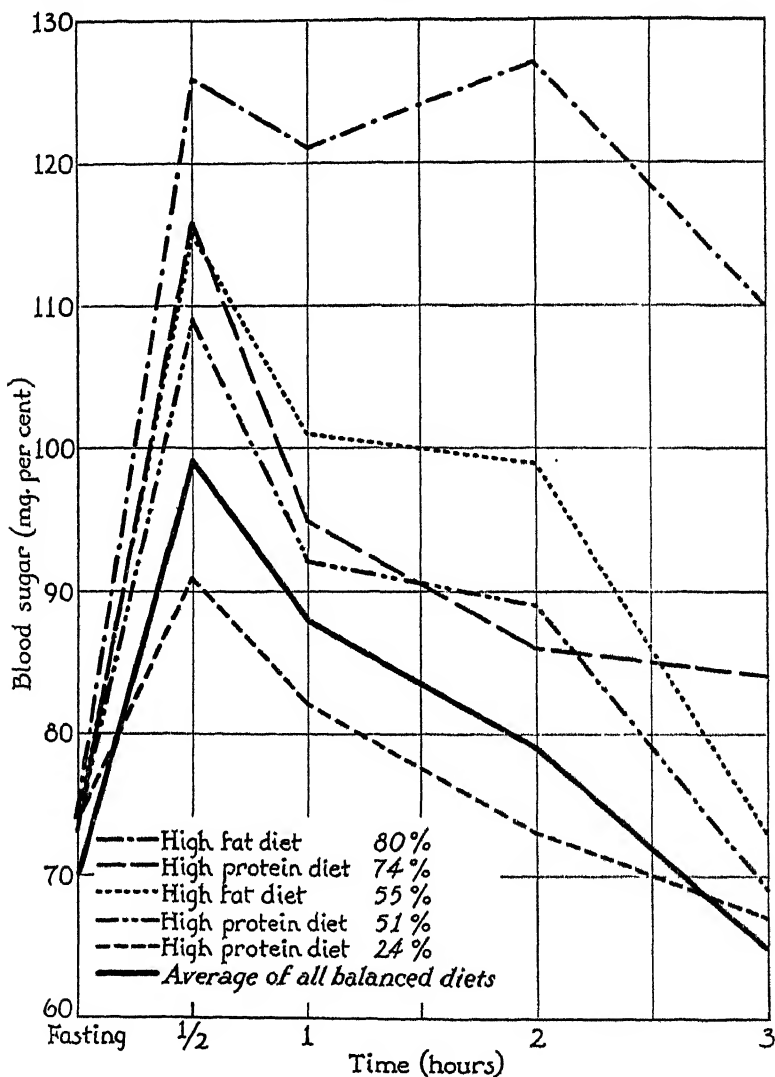


Fig. 3 Showing the effects of 55 and 80 per cent fat and 24, 51 and 74 per cent protein diets on glucose tolerance of six dogs. Second experimental period.

The results found on the high protein diet no. 1, on the high fat diet no. 1, and on the high carbohydrate diet are shown in figure 2; this covers the first experimental period.

The results found on the other experimental diets, used in the second period, are shown in figure 3.

#### DISCUSSION

In this dietary study an attempt was made to select materials which were suitable for dogs for the various diets. Since all were adult animals the aim was to hold the weight steady by giving the amount needed; the animals were weighed three times each week.

The balanced diet was relished by all the dogs; the high protein diet no. 1, in which granular gelatin was present, appeared distasteful to some of the dogs, but during that week the weather was unusually hot, so this factor may have had some influence. This was practically the only diet which was not completely eaten by all the animals. The high fat diets were very readily eaten.

During the first experimental period, the effects of high protein, high fat, and high carbohydrate diets were studied; the animal was placed back on the balanced diet after each experimental diet, before the next was tried. In the second experimental period, different percentages of high protein and high fat were studied, with a gradual increase to the maximum, and then a gradual decrease to the balanced diet.

It was necessary to use 3 gm. of glucose per kilogram of body weight for dogs, although 1 gm. per kilogram is sufficient for human beings. This was given by stomach tube; the passage of the tube did not seem to have an important effect on the level of the blood sugar; the average of fasting sugars for the six original dogs was 59 mg. per cent; 10 minutes after the passage of the tube, with introduction of water, the average was 62 mg. per cent.

In the tests after 7 days on the balanced diet, as shown in figure 1, there was little change in the fasting sugar, regardless of the type of diet which had been used before the

balanced. There is noted a tendency for the level at the half-hour to be higher, if high fat or high carbohydrate had been used before the balanced. This suggests that these diets leave some residual effect, which is noted at the half-hour period in the glucose tolerance test, even if a week of balanced diet has followed the experimental diet.

From figure 2 it will be noted that on high protein diet no. 1, there is a definite decrease in tolerance for glucose. This decreased tolerance is even more marked after high fat diet no. 1. The fasting level was slightly lower than the control, in both cases. With the high carbohydrate diet, the fasting level was higher, and the tolerance was increased; the results differed only slightly from those on the balanced diet.

In the second experimental period, as shown in figure 3, decreased tolerance after high protein was again noted; the higher the protein percentage in the diet, the greater the effect on the tolerance. The effect after 24.1 per cent protein was not noticeable.

With both 55 per cent and 80.1 per cent fat in the diet, a marked decrease in the tolerance for glucose was found.

From the results, one is justified in concluding that both increased protein and increased fat in the diet decrease the tolerance; in both cases, however, the carbohydrate in the diet is low.

There were great variations among the animals. Dog A showed the greatest effect as a decreased tolerance on the high fat diet. Dog B tended to show a delayed effect; this was most evident on the balanced diet after previous high fat and high carbohydrate diets. Dog C showed a delayed effect after the high fat diet; he showed a decreased tolerance on the high protein diets, but seemed gradually to overcome it. Dog D showed both an immediate and a delayed effect on the high protein and the high fat diets. She even showed a decreased tolerance on the high carbohydrate diet. Very great changes were noted in the blood sugar. Dog E behaved similarly to dog D. Dog F showed a decreased tolerance on

all the experimental diets. Dog G showed decreased tolerance on the high fat and high protein diets; the high carbohydrate was not done. Dog H showed a markedly decreased tolerance on high fat diets; this animal was a typical diabetic on the highest fat diet.

It is difficult to know how to interpret all the above findings. The differences in glucose tolerance are supposedly not due to differences in the rate of absorption. Trimble ('33) found glucose was absorbed from the gut of normal dogs at about the rate of 1 gm. per kilogram of body weight per hour.

Greenwald ('24) did not find a decreased tolerance after high protein diets, but did after high fat diets. du Vigneaud's ('25) findings agree with those of Greenwald. Malmros ('28) found low tolerance after low carbohydrate diets, and after high fat diets. He found that the decreased sensitivity toward glucose disappeared shortly after the return to the normal diet. He disagrees with other investigators in his results on high carbohydrate diets. Tolstoi ('29) found reduced tolerance after a high fat diet, with a return to normal tolerance within 2 weeks on a balanced diet. Sweeny ('27 and '28) found decreased tolerance after high protein and high fat diets, but increased tolerance after high carbohydrate diets. Himsworth ('33 and '34) found reduced glucose tolerance after high fat diets, and improved tolerance after a diet rich in carbohydrate. He considered the characteristic responses due to a change in the animal's susceptibility to insulin.

One should be exceedingly cautious in making general statements, since there is such variation in individual animals.

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## ADEQUACY OF SIMPLIFIED RATIONS FOR THE COMPLETE LIFE CYCLE OF THE CHICK

ALBERT G. HOGAN, ROBERT V. BOUCHER AND HARRY L. KEMPSTER

*Departments of Agricultural Chemistry and Poultry Husbandry, University of Missouri, Columbia*<sup>1</sup>

### ONE FIGURE

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So far as we are aware Osborne and Mendel ('18) were the first to achieve even the slightest success in rearing baby chicks on simplified diets. They used rations similar to those that had been found adequate for the rat, but it is evident from their brief description that these diets do not make even a close approach to supplying the minimum requirements of the chick. It is clear now that their difficulties were partly due to the fact that vitamin D had not been recognized at the time their observations were taken, but the attempts made after vitamin D was discovered were only slightly more successful than those made before.

Hogan, Guerrant and Kempster ('25) presented evidence that the failures were due solely to nutritional deficiencies, and this view was confirmed by Hogan and Shrewsbury ('30). More recently Hogan and Boucher ('33) succeeded in formulating a simplified ration that is adequate for growing chicks. It is quite evident now that the rations commonly used when the rat is the experimental animal are grossly inadequate for the chick, for it was not until drastic changes were made in the vitamin components that we were able to rear normal chicks consistently on simplified diets. No other successful attempt has come to our attention.

<sup>1</sup> Contribution from the Missouri Agricultural Experiment Station journal series no. 429.

In our more recent work the chicks were discarded at 6 weeks, for our earlier experience had demonstrated that when a ration is deficient practically all the abnormalities will develop within 4 weeks. It is well recognized though that 6 weeks is too short a period to establish the complete adequacy of a diet, so in order to provide a more rigorous test an attempt was made to rear chicks through successive generations.

#### EXPERIMENTAL

The experimental chicks were single comb White Leghorns. They were reared in battery type brooders, but as they approached maturity they were placed in wire cages 2 feet square. The floors of the brooder were wire screens, but the cages had floors of sheet metal, covered with a litter of sand. Both the brooders and cages were located in rooms that were equipped with thermostatic heat controls. The chicks were weighed, transferred to the brooders, fed and watered when 1 day old. The rations are described in table 1.

As will be shown later the rations were changed at frequent intervals. This was because our simplified diet was being developed at the time this investigation started, and as it was modified from time to time corresponding changes were introduced in the rations of the chicks that were reared to maturity. Later on as it became evident that the fertility of the eggs was low, numerous changes were made in an effort to correct that condition, but there is no reason to believe the rations were improved thereby.

Cockerel 994 and hens 1243 and 1297 represented the first generation of the first trial. They were hatched September 1, 1931, and given ration 1405 at once. Both pullets began laying at an early age, one at 162, the other at 164 days. The hens were mated with the cockerel, but the fertility of the eggs was low, and none hatched. After it seemed certain that living chicks could not be obtained from this group, the male was mated with a hen from the poultry farm, and hen 1297 was mated with a male from the farm. Living chicks were obtained from both crosses, showing that fertile chicks of both

TABLE 1  
Percentage composition of rations

	1405	1521	1552	1572	1601	1615	1668	1674	1713	1799	1801	1983	1996	2033
Casain <sup>1</sup>	35	35	35	35	35	20	20	35	35	35	35	35	35	35
Corn starch	35	17	29	29	30	45	42.6	27.6	30	28	27	21	19	25
Lard	3	5	7	7	11		11	11	11	11	7	5	5	6
Milk fat						11						6	6	6
Cellophane	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Salts <sup>2</sup>	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Calcium carbonate							1.3	1.3						
Monosodium phosphate							1.1	1.1						
Tricalcium phosphate														
Cod liver oil	2	2	2	1	1	1	1	1	1	1	1	1	1	3
Wheat germ oil <sup>3</sup>				1	1	1	1	1	1	1	1	1	1	1
Acid hydrolyzed yeast <sup>4</sup>	5	8	5	5	4	4	4	4	4	4	4	4	2	2
Water extract of yeast													4	4
Ether extract of egg yolk	10	8	6	6	2	2	2	2	2	2	6	6	6	6
Purified liver extract <sup>5</sup>	1	8	5	5	5	5	5	5	5	6	6	6	6	6
Liver extract residue <sup>6</sup>	1	8	3	3	3	3	3	3	3	4	4	4	4	4
Tikiki <sup>7</sup>	1	2	1	1	1	1	1	1	1	1	1	1	1	1

<sup>1</sup> Extracted with dilute acetic acid.

<sup>2</sup> Osborne and Mendel ('19).

<sup>3</sup> The ether extract of wheat germ.

<sup>4</sup> Prepared by hydrolyzing yeast with sulfuric acid.

<sup>5</sup> A commercial product used in the treatment of pernicious anemia. Supplied by Dr. David Klein of the Wilson Laboratories, Chicago, Ill.

<sup>6</sup> The fraction precipitated by 70 per cent alcohol during the manufacture of the Purified Liver Extract. See footnote 5 for source.

<sup>7</sup> Alcoholic extract of rice polishings, prepared by the method of Wells ('21).



sexes could be reared on the simplified diet, though the fertility was of a low order. The two chicks from hen 1297 were reared to maturity, and the pullet, no. 3472, laid her first egg at 152 days. She was mated with her full brother, no. 3471, and one chick was hatched, but it was subnormal in vigor and died the third day. When 202 days of age pullet 3472 became lethargic, and died 16 days later. Post-mortem examination<sup>2</sup> revealed a 152-gm. ovarian tumor. Doctor Durant, specialist in poultry diseases, informs us this condition is usually the result of a broken egg in the oviduct, and frequently occurs in commercial flocks. Death was, therefore, not necessarily the result of a nutritional deficiency nor of confinement under laboratory conditions. Until the time this tumor developed the hen was very active and indistinguishable from those reared normally. The male, no. 3471, was likewise normal and unusually belligerent. Both male and female had excellent plumage and very red combs and wattles.

Returning to the first generation group, all three were normal until March 22, 1932, when hen 1243 developed symptoms that resemble polyneuritis. The head retractions gradually became more severe, and death followed 8 days later. On July 20, 1932, mild head retractions were noticed in hen 1297, and this condition continued until September 2nd, when the observations on the first generation were discontinued. The decline in both these hens followed periods of active egg production, and the added demand during that period is believed to be the cause of the breakdown. The male, no. 994, was under observation continuously for 365 days. He received nothing but the simplified ration, was maintained constantly under laboratory conditions, and was normal throughout, except the toes were crooked.

After it became apparent that the first attempt to secure second generation chicks would probably fail, if the parent birds received simplified diets exclusively, a second trial was

<sup>2</sup> We are greatly indebted to Dr. A. J. Durant who made most of the post-mortem examinations.

started. The group included one male, no. 1862, and two females, nos. 1869 and 1880, and they were started at once on ration 1521. Hen 1880 began laying at 150 days of age, and laid a total of forty-eight eggs. Hen 1869 began laying at 154 days, and laid seventy-five eggs in all. Both hens were mated with cockerel 1862, and in each case living chicks were obtained. A total of eighteen were hatched and eleven survived. From this second generation, two males, nos. 101 and 4350, and two females, nos. 4347 and 4403, were then reared to maturity, in an attempt to obtain a third generation on a simplified diet.

When considered from all standpoints the record of hen 4403 is the best we have observed. She began laying at 170 days of age, and in the following 390 days she laid 249 eggs, a rate of 233 per year. However, she was obviously failing on the 568th day, and was killed when 585 days old. During the preceding 3 weeks she had lost approximately 600 gm. in weight. It developed at autopsy that a broken egg in the oviduct was the immediate cause of the decline.

In the meantime twenty-one of her chicks had been reared, and three were retained until they were mature. One of the females, no. 99, laid her first egg at the age of 169 days, and in the next 24 days she laid fifteen more. There was some indication when 181 days old that this pullet was failing, and she was killed on her 211th day. On post-mortem examination it was found that the duodenum was packed with sand. The other female, no. 100, was mated with a brother, no. 96, but the first attempt to secure living chicks failed. Few of the eggs were fertile and none hatched. It was thought the fertility of the male may have been of a low order, so he was replaced by a Rhode Island Red from the University Poultry Farm. Four living chicks were obtained from this cross and they were strong, vigorous, and grew rapidly on one of the simplified diets. They were discarded when 10 weeks old. The female was then remated with no. 96 and following this three eggs hatched. The chicks appeared normal at hatching and were above the average weight, but none survived longer

than 3 days. It is not believed that these deaths alone afford any positive evidence that the ration consumed by the mother was inadequate, as the conditions at that time were exceedingly unfavorable. The eggs were in the incubator during the last of July and first part of August, 1934, during the period of abnormally high temperatures. It is well recognized by poultrymen that chicks hatched during the summer are usually inferior. This hen began to decline at the age of 510 days, and died on her 528th day. The immediate cause of death was pneumonia, but the hen was excessively fat. Fatty degeneration of the liver, heart, and spleen were reported.

The male, no. 96, was killed and autopsied at the same time. He too was excessively fat, and the layer of adipose tissue in the posterior part of the body cavity was described as fully 1 inch thick. The liver had small indentations on the surface, but was not strikingly abnormal. The other organs seemed entirely normal.

The growth curves of the chicks retained until maturity are shown in figure 1, and their pedigrees are shown in table 2. The ration changes are shown in table 3.

In figure 1 the control weight-age curves were plotted from data published by Buckner, Wilkins and Castle ('18). Their chicks were reared under normal conditions on normal rations, and they grew more rapidly than any others reported, when corresponding numbers were involved. It is evident that, judged by any standard, the experimental chicks have grown at a satisfactory rate. The males grew normally and were apparently healthy throughout the period of observation. The females were normal until after they had been laying for varying intervals, but four out of six eventually developed some disease from which they did not recover.

Our chief interest was in the ability of the experimental chicks to reproduce, so a complete summary of the laying and incubation records is shown in table 4.

Even under the most favorable circumstances there is considerable variation in the fertility and viability of eggs, so

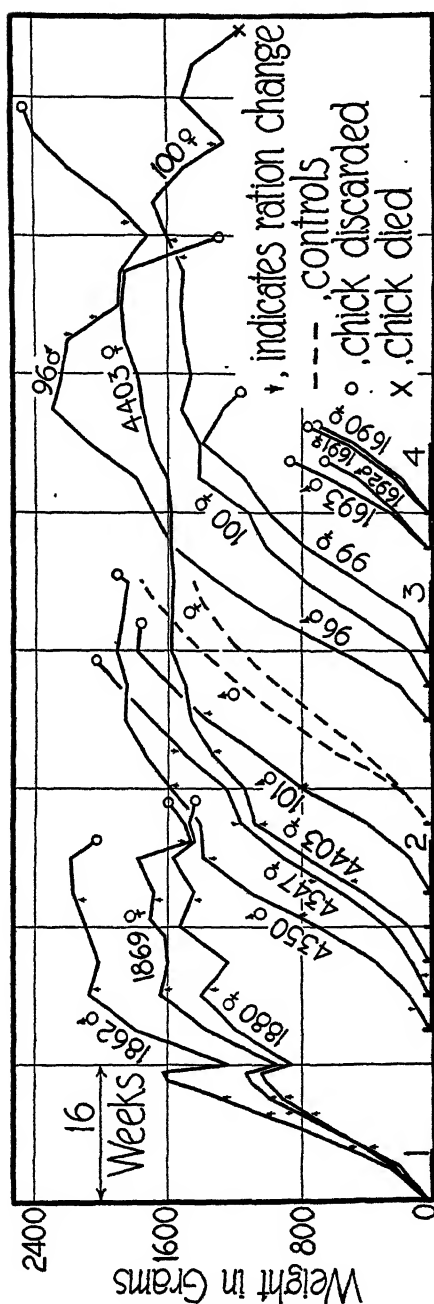


Fig. 1 Age-weight curves of four successive generations reared on simplified rations. The order in which they came is indicated by numerals at the base of the chart.

in one of the early trials it was decided to place commercial hatching eggs in the incubator as a control. These are compared with the total of experimental eggs in table 5.

Taking the data at their face value it must be concluded that the degree of success in rearing successive generations on the simplified diet was somewhat qualified. The fertility

TABLE 2  
*Relationship of chicks reared on simplified rations*

GENERATION	
1	1869F (2-1-32) <sup>1</sup> × 1862M (2-1-32) × 1880F (2-1-32)
2	$\frac{4347F (10-24-32) \ 4403F (11-21-32)}{\quad \quad \quad} \times \frac{4350M (10-24-32) \ 101M (12-24-32)}{\quad \quad \quad}$
3	99F (6-16-33) 96M (7-20-33) × 100F (6-16-33) × 1574M <sup>2</sup>
4	$\frac{1, 2, 3 (8-11-34)^3}{\quad \quad \quad} \quad \frac{1690F, 1691F, 1692M, 1693M (6-16-34)^4}{\quad \quad \quad}$

<sup>1</sup> Figures in parentheses indicate data of hatching.

<sup>2</sup> Rhode Island Red from Poultry Farm, reared on stock ration.

<sup>3</sup> None of these three survived longer than 3 days.

<sup>4</sup> All grew normally, were discarded at 10 weeks.

#### *Autopsy record*

1862M, killed 12-15-33. Normal.

1869F, 1880F, killed 1-13-33. Excessively fat, otherwise normal.

101M, killed 7-25-33. Normal.

4350M, killed 10-28-33. Normal.

4347F, killed 7-25-33. An 'internal layer.' May have been due to injury, as a broken egg. Twelve yolks were found. All organs normal.

4403F, killed 6-29-34. Decline due to broken egg.

96M, killed 11-25-34. Excessively fat. Liver had small indentations on surface. otherwise normal.

99F, killed 1-13-34. Duodenum impacted with sand.

100F, died 11-25-34. Fatty degeneration of liver, heart, spleen. Pneumonia immediate cause of death.

of the experimental eggs was less than half that of commercial eggs, though there was no marked difference in the hatchability of the fertile eggs.

It is impossible to determine from the available data whether or not the qualitative makeup of the ration was in any way responsible for the lowered fertility. Practically all

TABLE 3  
*Periods simplified rations were used*

DAYS <sup>1</sup>	RATION	DAYS <sup>1</sup>	RATION	DAYS <sup>1</sup>	RATION
Chicks 1862, 1869, 1880		Chicks 4347, 4350		Chick 96	
1	1521	1	1668	1	1801
45	1552	17	1674	309	1983
72	1572	94	1713	323	1996
113	1601	140	1799	401	2033
173	1615	199	1801		
247	1668				
Chick 101		Chick 4403		Chicks 99, 100	
1	1713	1	1674	1	1801
109	1799	66	1713	343	1983
186	1801	112	1799	357	1996
		171	1801	435	2033
				Chicks 1690, 1691, 1692, 1693	
				1	1996

<sup>1</sup> Age when the corresponding ration was first supplied.

TABLE 4  
*Egg records of hens reared on simplified rations*

HEN NO.	FIRST GENERATION		SECOND GENERATION		THIRD GENERATION	
	1869F <sup>1</sup>	1880F <sup>1</sup>	4403F <sup>1</sup>	4347F <sup>1</sup>	99F <sup>1</sup>	100F <sup>1</sup>
Days under observation	297	297	585	274	211	528
Age first egg laid, days	154	150	170	...	169	177
Number of eggs laid	75	48	249	...	16	90
Number of eggs incubated	73	48	135	...	0	64
Number of eggs fertile	28	20	64	...		17
Number of chicks hatched	7	11	31	...		7
Number of chicks reared	5	6	21			4

TABLE 5  
*Fertility and hatchability of experimental and of commercial eggs*

	SOURCE OF EGGS	
	Experimental	Commercial
	<i>per cent</i>	<i>per cent</i>
Fertility	40.3	89.0
Hatchability of fertile eggs	43.4	50.6

of the older birds were excessively fat, and this decreases the efficiency of breeding stock. There is also some uncertainty as to the effect of close confinement, and indoor conditions, on fertility. All the males have enormously developed combs and wattles, and this may be a by-product of some physiological disturbance of unexpected significance. Buckner, Insko and Martin ('32) made a detailed study of the effects of confinement on the growth and development of male chickens. They observed that when the cockerels were reared indoors their combs were about twice the normal size, the testes were about half the normal size, and their fertility was low. These authors also mention the fact that lack of exercise is not conducive to high fertility. Two years later Buckner, Martin and Insko ('34) emphasized again the depressing effect of close confinement on male fertility, and included some observations on the effects of closely confining the pullets. The hens that were reared in confinement were less productive than the controls, and their eggs were below the average weight. The control group with the highest production laid an average of 202 eggs their first laying year. There were no significant differences between the experimental and control hens in fertility or hatchability. The percentage of mortality was very heavy among the confined hens, as 58.5 per cent of them died. If the data of these investigators are taken as a standard of performance during confinement, then the adequacy of our simplified ration would seem to be demonstrated beyond question. According to this standard our chicks on simplified diets grew rapidly, were almost entirely free from abnormalities, and their egg production was satisfactory. Our best hen, no. 4403, laid at the rate of 233 eggs per year, and this is 15 per cent higher than the average production of the best laying controls reported by Buckner, Martin and Insko ('34).

The reports just cited make it seem probable that close confinement alone could explain our most serious difficulties, but as a matter of fact there were some additional complications. For example it is recognized by poultrymen that the

fertility and viability of eggs is highest in the spring. It was necessary for us to incubate the eggs throughout the year, and it was often necessary to hold them unreasonably long before placing them in the incubator. Furthermore, the original line had of necessity been closely inbred, and a genetic factor may have been partly responsible for the later failures.

It has been mentioned that the only serious difficulty encountered was the low fertility of the third generation, and the delay in obtaining the fourth generation chicks. At this same time our studies with growing chicks were still under way, and we were perplexed to find that the simplified diet had suddenly become less reliable. A majority of the chicks grew normally, but there was a definite increase in the incidence of leg weakness indicated by enlarged hocks, crooked leg bones, or slipped tendons. Many of the affected chicks were also subnormal in weight. We had previously regarded this ration as almost infallible, for during the preceding 2 years approximately 200 chicks were reared on this, or similar rations, with uniform success. The mortality had been 7 per cent, and the abnormalities of all types 8.1 per cent. The unexpected failures with growing chicks seemed to leave no doubt that the ration had become defective in some way, and it is reasonable to suppose that this weakness also had an adverse effect on the reproductive capacity of the mature individuals.

The source of these disturbances is not clear as yet, but various possibilities have been considered. It is well recognized that cod liver oil is toxic at times, and the supply then in use may have been inferior. A particular preparation of one of the other vitamin supplements may have been defective. A change in one of the original materials may have had an unfavorable effect on the inorganic constituents, especially of calcium and phosphorus, and for the present we have adopted this as our working hypothesis. Hunter and Funk ('30) stated some time ago that a maladjustment of these two elements increases the incidence of leg weakness, and this



observation has been confirmed repeatedly. Preliminary studies of the mineral content indicate that our recent difficulties will be explained in the same way.

In the course of our efforts to further standardize the preparation of the basal diet, various rations seemed promising. One of these, no. 2159, is described below.

*Ration 2159*

Casein	35	Wheat germ oil	4
Cornstarch	30.5	Acid hydrolyzed yeast	4
Cellophane	3	Liver extract	6
Lard	11	Tikitiki	2
Salt mixture <sup>3</sup>	4	CaCO <sub>3</sub>	0.5

In addition each chick receives daily 0.2 mg. crystalline carotene,<sup>4</sup> and 240 International units of irradiated ergosterol.<sup>5</sup>

As made up at present ration 2159 contains 0.7 per cent of calcium and 0.7 per cent of phosphorus. It has been used twice, and altogether twenty-four chicks received it as their sole diet. There were no mortalities and the rate of growth was satisfactory but there were two cases of slipped tendon, a rate of 8.3 per cent. Two or three similar rations have been equally satisfactory, and we feel they may be used with considerable confidence. We see no reason to doubt, therefore, that these simplified diets contain all nutrients that are required by the chick. It may be that some of them contain some nutrient in insufficient amount, or there may be some maladjustment between them. However, it is our view that the fundamental difficulties have been overcome. It may be an exacting task, but the way is open to determine qualitatively and quantitatively the nutritional requirements of the chick.

<sup>3</sup> Osborne and Mendel ('19).

<sup>4</sup> Purchased from S. M. A. Corporation, Cleveland, Ohio.

<sup>5</sup> Kindly supplied by Dr. C. E. Bill of Mead Johnson and Company.

## SUMMARY

1. Four successive generations of chicks have been reared on simplified rations.

2. All chicks grew rapidly. The males were normal in appearance throughout the period of observation but their fertility was low. The females were normal until they attained maturity, and their egg records compared favorably with those obtained under normal conditions. After periods of intensive egg laying the mortality of the hens was high.

3. Data published by other investigators indicate that the low fertility of the males and the high mortality of the females can be explained by the fact they were reared under laboratory conditions. If this explanation is correct the simplified diets are complete in all respects.

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# THE EFFECT OF 1 PER CENT COD LIVER OIL ON THE RAT, WITH PARTICULAR REFERENCE TO THE THYROID GLAND

CLAY B. FREUDENBERGER AND FRED W. CLAUSEN

*Department of Anatomy, University of Utah, Salt Lake City*

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Hamilton ('34) in comparing the thyroid weights of his control rats with the series of Freudenberger ('32) found that his weights were significantly lower. The stock diet fed these two series of animals differed only in that 1 per cent cod liver oil had been added between the time Freudenberger made his study and the time Hamilton did his work. Since Thomson ('32) found that 1 per cent cod liver oil was not protective against the goitrogenic activity of rachitogenic diets, Hamilton concluded that the marked variation between Freudenberger's series and his own was due chiefly to differences in technic of dissection. He felt that the possibility of actual changes in the thyroid weight of the colony in such a short time was rather slight.

Freudenberger and Billeter ('35) noted that the thyroid weights of their 6-month-old rats were smaller than those of Freudenberger's ('32) 3-month series. Freudenberger and Billeter were feeding their rats the same diet used by Hamilton.

These two instances led us to believe that the small thyroid weights might actually be due to the addition of the 1 per cent cod liver oil to the diet so this experiment was carried out to test this possibility.

## MATERIALS AND METHODS

Wistar albino rats of the female sex were used. They were weaned at 3 weeks of age and at once put on the diets. Twenty-five were given a constant supply of a well-balanced stock diet consisting of: casein, 15 per cent; whole milk powder, 10 per cent; sodium chloride, 0.8 per cent; calcium carbonate, 1.5 per cent; butter (unsalted), 5.2 per cent; and whole ground wheat, 67.5 per cent. Twenty-five litter mate controls were fed the same diet with the exception that 1 per cent pure cod liver oil (U.S.P.) was added. The diet was made up fresh at least every other day. All the animals were given excellent care which was similar in all cases. The care given was similar to that described fully by Freudenberger ('32).

The progress of each animal was carefully watched, and each animal was weighed weekly. At 4 months of age the animals were killed and autopsied according to the method of Jackson ('30). Both the nose-anus and tail lengths were measured. The head was weighed as well as the suprarenal glands, hypophysis, thyroid, thymus, and ovaries. The organs removed were fixed in Bouin's fluid. Sections of the thyroid gland, 10  $\mu$  in thickness, were stained with hematoxylin and eosin.

A comparison of the weights and measurements of the two groups of animals was made using modern biometrical methods. As is customary the difference between two means divided by the probable error of the difference is spoken of as the significance ratio in this paper.

## OBSERVATIONS

*Growth in body weight*

The growth of the two groups of rats was practically identical. Those not given cod liver oil averaged 36.8 gm. in weight on the day the experiment was begun while those given the oil averaged 36.1 gm. The average weekly weights of the two groups were practically the same throughout the

experiment. At the time of autopsy (table 1) the rats which were not given oil averaged 190.8 gm. in weight while those on oil averaged 191.1 gm. This slight difference is insignificant, the significance ratio being only 0.11. The addition of 1 per cent cod liver oil to the diet apparently does not affect growth in body weight.

TABLE 1  
*Statistical summary of results*

	MEAN		DIFFERENCE BETWEEN OIL AND NO OIL MEANS	DIFF. P.E. DIFF.
	Oil	No oil		
Body weight (gm.)	191.1 $\pm$ 2.3	190.8 $\pm$ 1.7	+ 0.3 $\pm$ 2.9	0.11
Nose-anus length (cm.)	19.91 $\pm$ 0.06	19.94 $\pm$ 0.06	- 0.04 $\pm$ 0.09	0.42
Tail length (cm.)	17.96 $\pm$ 0.11	17.93 $\pm$ 0.09	+ 0.03 $\pm$ 0.15	0.19
Head (gm.)	17.28 $\pm$ 0.14	17.37 $\pm$ 0.10	- 0.09 $\pm$ 0.17	0.52
Suprarenal glands (gm.)	0.0453 $\pm$ 0.0009	0.0466 $\pm$ 0.0008	- 0.0013 $\pm$ 0.0013	1.05
Hypophysis (gm.)	0.0105 $\pm$ 0.0003	0.0107 $\pm$ 0.0002	- 0.0002 $\pm$ 0.0003	0.54
Thyroid (gm.)	0.0169 $\pm$ 0.0002	0.0203 $\pm$ 0.0004	- 0.0034 $\pm$ 0.0004	7.72
Thymus (gm.)	0.4755 $\pm$ 0.0110	0.4610 $\pm$ 0.0092	+ 0.0145 $\pm$ 0.0144	1.01
Ovaries (gm.)	0.0578 $\pm$ 0.0011	0.0579 $\pm$ 0.0012	- 0.0001 $\pm$ 0.0016	0.07

### *Nose-anus length*

The rats not given oil had a final average nose-anus length (table 1) of 19.94 cm. while those which received the oil had an average length of 19.91 cm. The significance ratio for this slight difference is 0.42 so the difference must be regarded as insignificant.

### *Tail length*

The tail length (table 1) of the rats not fed oil averaged 17.93 cm. while those fed the oil had an average length of 17.96 cm. The significance ratio for this small difference is only 0.19 so the difference is not significant.

### *Occurrence of infections*

Although the gross appearance of the lungs was carefully noted in each case, practically no evidences of pathological changes were noted. In the case of one rat receiving the oil a small solid area of lung tissue was noted. The lungs of all the other animals appeared entirely normal.

The tympanic cavities were examined for pus. Pus was noted in one or both ears of eighteen of the rats not receiving the oil. Similar observations were made in the case of sixteen rats receiving the oil. The presence of the oil in the diet apparently did not significantly change the susceptibility of the rats to middle ear infection.

No other infections were noted in any of the rats studied.

### *Head weight*

The average weight of the head (table 1) of those rats not being fed oil was 17.37 gm. while the average weight of those being fed oil was 17.28 gm. Since the significance ratio for this difference is only 0.52, the difference can be regarded as being insignificant.

### *Weight of individual organs*

*Suprarenal glands.* The suprarenal glands (table 1) of those animals not receiving oil averaged 0.0466 gm. in weight while those of the animals receiving oil averaged 0.0453 gm. This difference is also insignificant since the significance ratio for the difference is only 1.05.

*Thyroid.* The average weight of the thyroid gland (table 1) in those animals not receiving oil was 0.0203 gm. while the average weight of the thyroid in those animals receiving oil was 0.0169 gm. The thyroid is, therefore, smaller in those animals receiving 1 per cent cod liver oil in their diet. The difference is unquestionably significant since the significance ratio is 7.72. The histological structure of the thyroid was studied in six animals of each group. No definite differences could be detected. Since such a great variability exists in

the normal histological structure of the rat thyroid, as has been recently pointed out by Coplan and Sampson ('35), it is extremely difficult to detect histological differences between the thyroid glands of two series of rats unless they are very well marked.

*Hypophysis.* No significant difference was found between the average weights of the hypophysis (table 1) of the two series of rats. The hypophyses of the rats not receiving oil averaged 0.0107 gm. in weight while those of the rats receiving oil averaged 0.0105 gm. The significance ratio for this difference is only 0.54.

*Thymus.* The average weight of the thymus (table 1) in those animals not receiving oil was 0.4610 gm. while that of those receiving the oil averaged 0.4655 gm. Since the significance ratio for this difference is only 1.01, the difference cannot be considered significant.

*Ovaries.* The average weights of the ovaries (table 1) of the two groups of rats were practically identical being 0.0579 gm. in the case of those not receiving oil and 0.0578 gm. in those receiving oil. The significance ratio is only 0.07.

#### DISCUSSION

It is interesting to note that the addition of 1 per cent cod liver oil to a normal well-balanced diet has no effect on growth in body weight or length, weight of head, weights of the suprarenal glands, hypophysis, thymus, or ovaries, but results in a significantly smaller thyroid. The incidence of infections was also the same in the two groups of rats. There would seem to be no evidence of a vitamin deficiency in the diet used even without the oil. Growth was good and the animals were remarkably free from all infections except of the middle ear. There was no evidence of xerophthalmia.

Sherwood, Toth and Carr ('34) found that feeding 1 to 5 cc. cod liver oil per kilogram body weight per day to rats produced a depletion of the colloidal content of the acini. They showed that an excess of vitamin D had no effect on the thyroid gland. Sherwood and Luckner ('35) found that ex-



cessive amounts of vitamin A in the diet of rats caused an irregular distribution to a depletion of the colloid of the thyroid gland. The cells lining the acini increased in height and a marked increase in stroma occurred. They found that iodine present in cod liver oil was not responsible for any change in the thyroid gland, but that iodine in the approximate amount found in cod liver oil, given in the form of potassium iodide produced an increase in colloid after 18 days. The acini were strikingly distended and composed of extremely low epithelium.

Coplan and Sampson ('35) found that an iodine deficiency in the diet of albino rats produced an initial hypertrophy of the thyroid gland in both males and females and that this persisted for a longer time in females. It was followed in both sexes by atrophy. They found that vitamin A deficiency produced definite hypertrophy in the female, but consistent atrophy in the male. A deficiency of iodine and vitamin A in the diet produced an initial hypertrophy in males and females. In males atrophy followed with continued deficiency. Coplan and Sampson have reviewed the literature in regard to the effects of diets deficient in iodine on the thyroid gland. They also call attention to the literature in regard to other dietary defects (vitamin A, vitamin D, excess of calcium) on the thyroid gland. Since this has been done so recently, very brief mention of the literature will be made in this paper.

As far as we are aware there is no evidence in favor of our diet without the cod liver oil being deficient in either vitamins or iodine. It is, therefore, interesting to note that the addition of 1 per cent cod liver oil to the diet resulted in a smaller thyroid gland with no other changes that could be detected in the animals. It would seem that the thyroid gland is the organ which is most sensitive to cod liver oil. Agduhr ('29) showed that regressive tissue changes were produced with large doses of cod liver oil. It is, however, clear that no such changes were produced in this experiment.

All experimental workers using albino rats are very interested in normal organ weights. The results in this experi-

ment bring up the interesting question of just what is the normal thyroid weight. Is it the weight as found in those rats without the oil or that of those which received oil in their diet?

#### SUMMARY

Twenty-five female Wistar albino rats have been fed a well-balanced stock diet from 3 weeks of age to 4 months of age while a similar group has been fed the same diet with 1 per cent cod liver oil added. All the animals were killed and autopsied at 4 months of age. No difference was found between the groups in body growth, body weight, nose-anus length, tail length, incidence of infections, or in the weights of the head, suprarenal glands, hypophysis, thymus or ovaries. The thyroid gland was found to be significantly smaller in those rats which received the diet containing the cod liver oil.

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# THE RELATION OF THE RATE OF GROWTH TO DIET<sup>1</sup>

## III. A COMPARISON OF STOCK RATIONS USED IN THE BREEDING COLONY AT THE CONNECTICUT AGRICULTURAL EXPERIMENT STATION

LAFAYETTE B. MENDEL AND REBECCA B. HUBBELL

WITH THE ASSISTANCE OF LUVA FRANCIS

*Biochemical Laboratory, Connecticut Agricultural Experiment Station, and  
Department of Physiological Chemistry, Yale University, New Haven*

ONE FIGURE

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During the last 20 years, numerous workers have reported progressive increases in the average growth rate of the albino rat in response to selective breeding or to improved dietary conditions. References to some of these observations have been made by Osborne and Mendel ('26) and by Anderson and Smith ('32).

It is well known that the animals in the colony at the Connecticut Agricultural Experiment Station have been bred for about 25 years without accessions from elsewhere. Any changes in rate of growth or general condition of the rats during this period should, therefore, be attributed to the management of the colony. In 1926, in connection with a study of the factors that influence the rate of development, Osborne and Mendel ('26) recorded growth curves for males of the colony for 1912, 1919 and 1925. They also showed that rats fed a special ration, relatively high in protein, grew at the rate of 4.0 gm. a day for the period from weaning to

<sup>1</sup>The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

100 days of age, as contrasted with 2.1 gm. for stock males. This observation, followed by the work of Anderson and Smith ('32), led to the adoption of a new stock ration which has been in use since 1931. The accelerated growth rate, first observed by Osborne and Mendel for a few animals, has been maintained as a regular characteristic of the colony for more than ten generations. It seems of interest, therefore, to record at this time some of the data regarding the animals of the colony as it is today, together with a brief account of its history prior to 1931.

From the dietary standpoint, we recognize three periods, and for convenience designate them as 1912, 1919 and 1935. For the first of these periods, 1912, the stock ration consisted of an abundance of 'Old Grist Mill' dog biscuit and sunflower seeds, with carrots and other fresh vegetables two or three times a week. Rock salt was always kept in the cage. After October 1912, nursing mothers and young rats received fresh milk or 'milk food' in addition. The records do not indicate how long the milk was supplied after the young were weaned. During the second period, beginning in 1915, a special brand of dog biscuit<sup>2</sup> was used. This was supplemented with fresh vegetables, usually carrots. Nursing mothers and young rats under 4 or 5 weeks of age received in addition a 'milk food' paste consisting of milk powder 60 per cent, starch 12 per cent, and lard 28 per cent (Ferry, '20). The ration in use now is a modification of that devised by Anderson and Smith as a basis for their study of the effect of mating interval on reproductive performance (unpublished data). It consists of G.L.F. calf meal (Maynard, '30) 97 per cent and cod liver oil 3 per cent.<sup>3</sup> Nursing mothers and young rats under 6 weeks of age receive in addition a 'paste food' consisting of casein 25 per cent, whole milk powder 25 per cent, wheat embryo 20 per cent, and lard 30 per cent.

<sup>2</sup> Special dog biscuit composed of wheat, oats, beef, rice, corn-meal and cottonseed oil, made by Potter-Wrightington, Inc., Boston.

<sup>3</sup> The formula has been modified recently in accordance with a change in the commercial calf meal. This product now contains 5 parts in 2000 of "cod liver oil reinforced in vitamin D."

The calf meal food and 'paste food' are both supplied *ad libitum*. Each rat also receives 1 gm. of dried yeast daily except Sunday, and those without 'paste food' are given 3 gm. of wheat embryo per week. No 'green' food of any sort is used.

Rats are first bred at 120 days of age, according to the general procedure described by Ferry ('20). Each female is permitted three matings, with an interval of 3 weeks between weaning and subsequent remating. Litters are reduced to eight rats at birth, and the young are weaned at 21 days of age. The stock colony is maintained by selecting young from first, second or third litters. No female is mated more than once with the same male. Our records include the number of young cast, weights of the young at birth, at weaning, and at weekly intervals thereafter until the animals are terminated. Unfortunately, for the sake of comparison, the earlier records are not so detailed. However, we have been able to secure from them data which may be considered representative of the earlier dietary procedures. The significant facts are summarized in figure 1 and table 1.

Figure 1 records the growth of male rats from weaning to 280 days, and of females until they are mated at 120 days. For both sexes there was a gradual increase in growth rate from 1912 to 1925. This was probably due both to improvement of the stock through careful breeding, and to the inclusion in the ration after 1912 of the 'milk food,' which was doubtless more easily utilized by the young animals. In the light of the very successful experiments of Osborne and Mendel in 1926, in which growth was accelerated by the use of diets that contained a higher percentage of protein than had previously been considered desirable, we may also attribute the increased growth rate in 1919 and 1925 over that in 1912 to the fact that the rations after 1915 supplied an increased proportion of total calories from protein, approximately 18 per cent as contrasted with 13 per cent in the ration used in 1912. Whether the more marked change in growth noted between 1925 and 1935 can be attributed to a further

increase in protein has not been fully determined. In the present ration, approximately 23 per cent of the total calories is supplied by protein. The marked acceleration in weight increase occurs in the period from weaning to 100 days of age. Subsequently the 1925 and 1935 curves are almost identical.

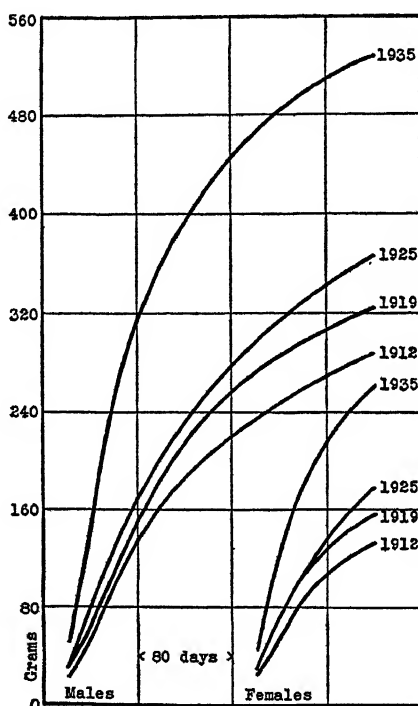


Fig. 1 Growth of stock rats.

TABLE 1

*Summary of reproductive performance under different dietary conditions*

	PERCENTAGE OF FERTILE MATINGS	NUMBER CAST	WEIGHT AT BIRTH	PERCENTAGE WEANED	WEIGHT AT WEANING		DAILY GAIN TO 100 DAYS	
					Males	Females	Males	Females
			gm.		gm.	gm.	gm.	gm.
1912	86	7.2	...	71	23	26	1.8	1.2
1919	65	6.3	...	67	31	31	2.0	1.4
1925	68	6.4	...	76	31	30	2.1	1.6
1935	93	9.6	5.8	90	48	47	4.0	2.5

Figures are averages of rats in the colony during the year indicated.

We have been inclined to attribute the rapid development during the first 6 weeks of life to the use of 'paste food' for the nursing mothers and young rats. That this is one factor is shown by a comparison of our weight gains with those reported by Anderson and Smith ('32) who used the same combination of calf meal food and 'paste food,' but with the latter available at all times, instead of only during the first 6 weeks. On this regimen, their rats maintained the increased growth rate over longer periods than do our animals. These authors recorded the average time for male rats to grow from 60 to 200 gm., to 300 gm., to 400 gm., and to 500 gm., respectively. The gain to 200 gm. was made in 23.3 days; our animals require 25 days for the same increase. With an average weaning weight of 48 gm., our rats would receive 'paste food' for only 19 or 20 days out of the total period required to make this gain. For the second interval, from 60 to 300 gm., the difference is more marked, namely 41.1 days for the Anderson and Smith rats, while our time is 51 days. For the two other intervals indicated, the gap is still wider, being 69.1 days as contrasted with 100 days for the gain from 60 to 400 gm., and 102.4 days in comparison with 197 days to attain the maximum weight discussed in their report.

The use of a 'paste food' is not the only factor, however, which may be regarded as responsible for the superior rate of growth in this colony in 1935 as compared with former years. A somewhat similar concentrated food was in use from 1912 until 1931 when the present regimen was initiated. The 'paste food' in use now supplies more protein than did the old 'milk food' and also includes 20 per cent of wheat embryo. A limited number of observations indicates that the calf meal food is itself in part responsible for the change in growth rate. We have observed the growth of male rats without the use of 'paste food' subsequent to weaning. Despite the fact that such growth is inferior to that observed with the more complex ration, it is considerably above that obtained under the old dog biscuit-milk food regimen. We feel, therefore, that both the calf meal food and the paste food contribute



to the present improved rate of growth. According to our analyses, this ration is richer in protein than the older one, and definite amounts of dried yeast and wheat embryo are fed separately, thus ensuring, with the cod liver oil included in the calf meal food, a liberal supply of 'accessory food substances,' even though no 'green' food is used.

Data for the colony at different periods in its history, summarized in table 1, afford evidence of the effectiveness of the present ration for reproduction, as judged by the number and size of litters, percentage weaned, and weights of the young at birth and at weaning. The figures for 1912 indicate a better record for number of young cast and weaned than in the years immediately following. It will be noted, however, that the animals were small at weaning. In the early records, there is frequent mention of the fact that nursing mothers were given milk, and bread and milk, "to see if the young would grow any better." That such additions were successful is shown by the fact that after 1912 fresh milk or 'milk food' was supplied regularly to nursing mothers and young rats. As a result, the young attained weights at weaning which were appreciably better as is shown by the 1919 record. During this same period, however, there was a reduction in the percentage of fertile matings. We have no explanation for this decrease.

We have included data for 1925 in the table in order to show the behavior of the stock animals at the time the rapid growth studies were initiated (Osborne and Mendel, '26). It is clear that there had been no marked increase in number and size of litters, or in rate of growth, as a result of careful selection of vigorous animals for breeding. If the type of growth now observed had been induced largely through selection, we feel that the upward trend would have been more marked in 1925, after the ration had been in use for 10 years. After the adoption of the new stock food in 1931, however, there was a marked improvement in reproductive performance and in rate of growth. These changes were evident immediately and have been maintained through succeeding generations.

## SUMMARY

A comparison has been made of growth and reproductive performance of the albino rat during three periods in the history of the colony at the Connecticut Agricultural Experiment Station. A gradual increase in rate of growth has been noted during the entire 25 years. This increase was very marked when a change of ration was made in 1931. The improved growth rate has been accompanied by superior reproductive performance.

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# THE COMPARATIVE RACHITOGENIC PROPERTY OF OATS AND CORN<sup>1</sup>

LAWRENCE L. LACHAT AND LEROY S. PALMER

*Section of Animal Nutrition, Division of Agricultural Biochemistry,  
University of Minnesota, St. Paul*

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The faulty skeletal development and poor teeth of animals (chiefly dogs) fed cereal diets were first emphasized by E. Mellanby ('18) and May Mellanby ('18). Later work led E. Mellanby ('26) to postulate the existence in cereals of definite rachitogenic substances or antivitamin which he called toxamins, the exact nature of which has not yet been determined. Mirvish ('29, '30) concluded that he had been able to concentrate a rachitogenic toxamin from cereals, especially from oatmeal. He separated an alcohol soluble fraction which lowered blood calcium when injected into the circulation of mature rabbits. King and Hall ('31), using chicks, also have concluded that oatmeal probably contains a specific rachitogenic substance.

The rachitogenic effect of cereals, especially oats, has been explained on other grounds than the presence of toxamin. Fine ('30) concludes that any difference between cereals is due to difference in vitamin D content, while Bruce and Callow ('34) believe that the inferiority of oatmeal is due to the lower availability of its phosphorus, present chiefly as salts of phytin (inositol hexaphosphate). However, Harris and Bunker ('35) using rats, failed to find any correlation between the rachitogenic properties of different samples of corn and either the absolute or relative amounts of phytin phosphorus present.

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On the other hand Booth, Henry and Kon ('35) report data for rats fed rachitogenic corn rations which are believed to support the conclusions of Bruce and Callow.

Steenbock, Black and Thomas ('27, '30) in extensive studies with rats and dogs failed to secure evidence of greater rachitogenic influence of oats than of corn, although both of these cereals seemed to be less antirachitic than wheat. de Wildt and Brouwer ('32), likewise, found corn to be more rachitogenic than oats, and more so than barley, rye or wheat. Steenbock and Kletzien ('31) were unable to isolate from cereals any material possessing rachitogenic properties.

The data of King and Hall ('31) are the only ones which have been interpreted in favor of the existence of rachitogenic substances in cereals which lend themselves to statistical treatment. Such a study of the data from their birds receiving the basal ration and their birds receiving the basal ration plus 20 per cent oat groats shows high variability in both groups and only a moderately significant difference between the means of the two lots ( $P = 0.05-0.02$ ). The importance of this value is decreased somewhat because of poor control of other factors in the rations fed, other than a possible unidentified toxamin.

#### EXPERIMENTAL

This paper presents certain phases of a study of the comparative rachitogenic property of oats and corn. The experiments reported include, 1) a repetition of certain of the King and Hall experiments with chicks, in comparison with rations containing a higher proportion of oats and in which all important factors were controlled except the possible toxamin, 2) attempts to concentrate a rachitogenic substance from oat groats and demonstrate its activity, 3) tests of rachitogenic diets indicating that the severity of the rickets produced has a bearing on the demonstration of rachitogenic differences between oats and corn. Both rats and chicks were employed because of the recognized quantitative differences between the etiological factors concerned in the production of rickets in

these species. The relatively greater requirement for vitamin D by chicks than by rats was considered advantageous and the close analogy between infantile rickets and rat rickets was considered justification for employing rats in this study.

The chicks used were day-old, single comb White Leghorns hatched from eggs produced at the Minnesota Agricultural Experiment Station. Carefully selected groups were housed in metal, electrically heated battery brooders and maintained on raised screens of coarse mesh. Group food consumptions and individual weights were recorded at weekly intervals and in most experiments the food consumption of the comparative groups was equalized. In all experiments the calcium, phosphorus and nitrogen contents of the comparative rations were also equalized following a preliminary chemical analysis of each feedstuff employed. The rachitogenic property of the rations was judged by the ash content of the left tibia and fibula, after drying and extraction with alcohol and ether.

The rats employed were 28-day-old closely inbred stock reared in this laboratory from mothers fed a modified (higher protein) Steenbock stock diet for production or rats suitable for vitamin D assay. They were housed individually in metal cages on screen floors, care being taken to distribute litter mates as equally as possible among the different experimental groups simultaneously on test. Rachitogenic effect was judged by the ash content of the right and left femurs of each animal, after drying and extraction of lipides.

Nitrogen in feedstuffs was determined by the Kjeldahl method, calcium and phosphorus by the method of Morris, Nelson and Palmer ('31), and water-soluble chloride volumetrically.

#### *Repetition of King-Hall experiments*

King and Hall compared the addition of 20 per cent oat groats and oat groats plus vitamin D supplements or carotene to a well-balanced basal ration, approximately 78 per cent of which was corn and wheat. We employed the same basal

ration in a similar study, adding oat groats and also oat groats plus cod liver oil concentrate of known vitamin D potency in comparative tests as shown in table 1. Two additional rations were tested in comparison with the foregoing. These differed from those employed by King and Hall in that oat groats replaced the corn meal in order to secure a higher concentration of oats; the ration of one of these additional groups contained cod liver oil concentrate, the other did not (groups 3 and 6). Table 1 shows the mean bone ash of the various groups after 8 weeks' feeding. The statistical analysis of the data does not indicate that under the conditions employed oat groats was more rachitogenic for chicks than yellow corn meal.

#### *Attempts to concentrate rachitogenic substance*

Oatmeal extracts were prepared essentially by the method described by Mirvish ('30), some of the steps being facilitated by semi-plant scale apparatus available in this laboratory. In general the procedure involved 1) two to four successive extractions with 0.5 per cent HCl solutions at room temperatures, 2) one or two successive extractions with hot (80°C. to boiling) 0.5 per cent HCl solution, 3) clarification of the combined extracts by passage through a Sharples supercentrifuge, 4) neutralization with NaOH solution, 5) concentration at reduced pressure, 6) clarification by means of the supercentrifuge, 7) further concentration to a syrup in a warm (70°C.) air chamber, 8) several successive 24- to 48-hour dialyses in cellophane bags against large volumes of distilled water, 9) concentration of the combined diffusates to a viscous mass, 10) repeated extractions of this mass with boiling 95 per cent ethanol, 11) clarification of the combined alcohol extracts by filtration through glass wool.

Four different preparations were made involving some differences in procedure in steps (1) and (2). For example the HCl extractions, first carried out in muslin bags, were later made by suspending the oatmeal directly in the acid and stirring, either continuously by mechanical stirrer for step (1) or intermittently by hand in step (2). The extracts were

TABLE 1  
*Bone ash of chicks fed rations similar to those employed by King and Hall,<sup>1</sup> with statistical study of data*

GROUP	NUMBER OF CHICKS	SUPPLEMENT TO BASAL RATION	BONE ASH		DIFFERENCE BETWEEN MEANS OF GROUPS	P <sup>2</sup>	STATISTICAL SIGNIFICANCE
			Mean	S. E.			
1	14	1 per cent corn oil	<i>per cent</i> 36.4 ± 0.75	<i>per cent</i> 7.72			
2	10	20 per cent oat groats plus 1 per cent corn oil	35.5 ± 0.80	7.10	1-2 = 0.9	0.5-0.4	Negative
3	10	20 per cent oat groats replacing 20 per cent corn meal 1 per cent corn oil added	38.2 ± 1.09	9.04	3-1 = 1.8	0.2-0.1	Negative
4	12	0.03 per cent cod liver oil concentrate <sup>3</sup>	44.1 ± 1.59	12.50	4-5 = 3.3	0.2-0.1	Negative
5	14	20 per cent oat groats plus 0.03 per cent cod liver oil concentrate <sup>3</sup>	40.8 ± 1.27	11.63			
6	13	20 per cent oat groats replacing 20 per cent corn meal. 0.03 per cent cod liver oil concentrate <sup>3</sup> added	42.5 ± 1.05	8.93	4-6 = 1.5	0.5-0.4	Negative

<sup>1</sup> King and Hall employed Barred Rocks in their experiments but it seems unlikely that the breed of fowl could be a factor in experiments of this character.

<sup>2</sup> Probability.

<sup>3</sup> The cod liver oil concentrate was diluted with corn oil, the total oil addition being 1 per cent.



syphoned off after settling. In the later extractions boiling was not resorted to; instead porcelain-lined steel drums containing the groats and solvent were immersed in boiling water.

The first two concentrates A and B were found to give positive tests for sugars (Molish test) sterols (faint Whitby-Salkowski test) and basic substances precipitable by phosphotungstic acid. Quantitative analysis showed that 22 to 50 per cent of the original Ca and 2.0 to 5.0 per cent of the original P of the oatmeal had been recovered in these concentrates.

These concentrates and also concentrates C and D, which were prepared by using less than boiling temperatures in step (2), were imposed on rachitogenic rat and chick diets, containing chiefly yellow corn, and their rachitogenic effects tested on these species in comparison with the untreated rations. The amount of concentrate added was equivalent to 200 per cent oatmeal. Similar tests were made with chicks using the material thrown out by the supercentrifuge in each of the two steps in which the centrifuge was employed, as well as the material which failed to dialyze. Data are not presented for the tests on concentrate D using rats and chicks or on any of the other materials recovered for which chicks were employed, since in these tests the preparations proved toxic<sup>2</sup> to the animals, probably because of their high NaCl content.<sup>3</sup> In this respect the results were contrary to those reported by György et al. ('33) who conclude that the high proportion of NaCl resulting from the subsequent neutralization of cereals treated with HCl exerts an anti-rachitic effect when such products are tested biologically.

The results obtained by imposing concentrates A and B on a rachitogenic yellow corn diet for rats and concentrate C on a rachitogenic yellow corn diet for chicks are presented in table 2. The rat tests lasted 21 days and the chick tests 14 days. Extract C was not tested with rats.

<sup>2</sup> The rats lost weight, became highly irritable and savage and died suddenly at an early date. More mature rats behaved similarly but survived longer. The chicks also lost their appetite and only a few survived the experimental period.

<sup>3</sup> Extract D contained 6 per cent NaCl and the chick rations containing the other materials recovered contained from 2.5 to 8.5 per cent NaCl.

TABLE 2  
*Bone ash of rats and chicks fed rachitogenic corn diets with and without oat concentrate (equivalent to 200 per cent oatmeal) imposed, with statistical analysis*

SPECIES	NUMBER OF ANIMALS	RATION	BONE ASH			DIFFERENCE BETWEEN MEANS OF GROUPS	P <sup>2</sup>	STATISTICAL SIGNIFICANCE
			Mean <sup>1</sup>	S. E.	C. V.			
Rat	10	Low Ca	per cent		per cent			
Rat	8	Low Ca + con. A	46.6 ± 0.79 (1)		5.34			
			43.8 ± 0.60 (0)		3.88	2.8	0.02-0.01	Positive
Rat	9	Med. Ca	26.3 ± 0.95 (1)		10.81			
Rat	9	Med. Ca + con. B	28.2 ± 1.09 (1)		11.58	1.9	0.2-0.1	Negative
Rat	10	High Ca	27.0 ± 0.75 (0)		8.80			
Rat	10	High Ca + con. B	27.9 ± 0.74 (0)		8.34	0.9	0.4-0.5	Negative
Chick	33	Basal	37.0 ± 0.40 (7)		6.20			
Chick	13	Basal + con. C	36.6 ± 0.71 (2)		2.88	0.4	0.6-0.5	Negative

<sup>1</sup> Based on surviving animals, mortality being shown in parenthesis.

<sup>2</sup> Probability.

In the study of concentrate A, using rats, the basal rachitogenic diets were in part modifications of the Steenbock-Black ('24) diet, only 1 or 2 per cent  $\text{CaCO}_3$  being added respectively in two of the diets instead of the prescribed 3 per cent, the purpose of the modifications being to produce several different degrees of rickets in order to bring out more clearly the presence of rachitogenic substances in the concentrate. The modified rations thus consisted of  $\text{CaCO}_3$  1, 2 or 3 parts,  $\text{NaCl}$  1 part, commercial wheat gluten 20 parts and yellow corn to make 100 parts. The lowest Ca ration contained approximately 0.41 per cent Ca and 0.34 per cent P. When the oat extract A was imposed on it these values increased to 0.48 and 0.35 per cent Ca and P, respectively.

The rachitogenic ration given the chicks was a modification of the Hart-Kline-Keenan ('31) ration consisting of yellow corn 70 parts, wheat flour middlings 10 parts, crude domestic casein 12 parts, alfalfa leaf meal 3 parts, whole dried yeast 2 parts,  $\text{Ca}_3(\text{PO}_4)_2$  1 part,  $\text{CaCO}_3$  1 part,  $\text{NaCl}$  1 part. This ration contained 3.05 per cent N, 0.94 per cent Na and 0.78 per cent P. When the oat extract C was imposed the yellow corn was reduced to 64 parts, the  $\text{Ca}_3(\text{PO}_4)_2$  reduced to 1.54 parts and the  $\text{CaCO}_3$  reduced to 0.74 parts. This ration contained 2.92 per cent N, 0.85 per cent Ca and 0.68 per cent P, essentially the same as the basal ration. The rations were also essentially isodynamic. The group food consumption of the chicks in the two lots was also equalized by keeping close record of the daily food consumption. The mortality was seven out of twenty birds in the group fed the oat extract.

The data in table 2 show that oats concentrate A was definitely rachitogenic for the rats when imposed on the Steenbock-Black diet containing only 1 per cent added  $\text{CaCO}_3$  but that concentrate B did not show this effect when imposed on a like ration containing higher concentrations of Ca. Also, concentrate C was without effect when imposed on the rachitogenic chick ration. Unfortunately there was not sufficient of the concentrate C to repeat the rat experiment using the low Ca diet. It is possible that the milder heat treatment

employed for the preparation of this concentrate failed to secure the rachitogenic substance or that the different lot of oatmeal employed lacked the principle. Of the two explanations the former appears more plausible in view of other experiments with chicks in which this lot of oatmeal was more rachitogenic than corn. The possibility of chicks being less susceptible to rachitogenic substance must be discounted in view of the results presented below.

Consideration of the rat data alone suggests that if concentrate B, which was made almost exactly like concentrate A, contained the active principle it probably could not be expected to lower still further the ash of bones already rendered so strongly rachitic by the basal ration. There should be a limit to the severity of decalcification (of lack of calcification) caused by a rachitogenic diet. It is unfortunate that insufficient material prevented a test of the effect of concentrate B on chicks and on rats fed the low Ca ration (containing 1 per cent  $\text{CaCO}_3$ ).

The suggestion that a rachitogenic principle becomes evident in these types of experiments only when the basal ration is itself only mildly rickets producing is strengthened by the following experiments conducted on chicks.

*Relation of severity of rickets to demonstration of rachitogenic principle*

The modification of Hart-Kline-Keenan ('31) rachitogenic ration for chicks, described above, gives satisfactory growth and calcification when fortified with adequate vitamin D, but alone ordinarily produces severe rickets in young chicks in the course of a few weeks. Since this ration contains 70 per cent yellow corn it would seem to be ideal for comparison of rachitogenic properties of different cereals which may be substituted in toto for the yellow corn. Moreover, protein (casein), vitamin (yeast and alfalfa leaf meal) and mineral ( $(\text{Ca}_3\text{PO}_4)_2$  and  $\text{CaCO}_3$ ) additions make it possible to adjust the N, Ca and P contents of such comparative cereal rations so that no known major quantitative differences exist between

TABLE 3  
*Bone ash of chicks fed yellow corn vs. rolled oats rachitogenic rations and statistical significance of results*

EXPERIMENT NO.	DATE OF EXPERIMENT	NUMBER OF CHICKS	RATION	LENGTH OF EXPERIMENT	BONE ASH		DIFFERENCE BETWEEN MEAN ASH	P <sup>4</sup>	STATISTICAL SIGNIFICANCE
					Mean <sup>1</sup> S. E.	O. V.			
				<i>weeks</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>		
1	Aug. 1932	35	Yellow corn	3	36.9 ± 0.42(3)	6.45	—		
1	Aug. 1932	34	Rollod oats	3	31.0 ± 0.61(10)	9.68	— 5.9	Less than 0.01	Positive
2	Nov. 1932	13	Yellow corn	2	33.3 ± 0.61(0)	6.59	—		
2	Nov. 1932	10	Rollod oats	2	31.5 ± 0.89(0)	8.90	— 1.8	0.1	Negative
3a	Feb. 1933	14	Yellow corn	2	29.3 ± 0.82(4) <sup>2</sup>	8.79	—		
3a	Feb. 1933	21	Rollod oats	2	28.3 ± 0.71(11) <sup>2</sup>	7.90	— 1.0	0.6-0.7	Negative
3b	Feb. 1933	14	Yellow corn	3	29.7 ± 0.78(3) <sup>2</sup>	8.67	—		
3b	Feb. 1933	14	Rollod oats	3	28.9 ± 1.15(6) <sup>2</sup>	11.27	— 0.8	0.5-0.6	Negative
4	May 1933	35	Yellow corn	2	37.0 ± 0.40(2)	6.20	—		
4	May 1933	47	Rollod oats	2	32.0 ± 0.38(6)	7.64	— 5.0	Less than 0.01	Positive

<sup>1</sup> Based on chicks which survived, the mortality being shown in parenthesis.

<sup>2</sup> Birds dying during first 2 weeks.

<sup>3</sup> Birds dying during third week.

<sup>4</sup> Probability.

them. Besides, such qualitative differences in vitamins A and B (complex) as do exist cannot be regarded as rendering any of them inadequate in these respects.

On the above basis several comparisons were made between rations containing 70 per cent yellow corn meal and 70 per cent rolled oats. Not all of these comparisons were made at the same time although experiments 1, 2 and 3 (a and b) were carried out using rations made at a single mixing. The rations for experiment 4 were mixed at a later date. The results are shown in table 3. Experiments 3a and 3b are the same experiment, one-half of the birds in each group being sacrificed at the end of 2 weeks, the remainder being continued to the end of the third week. In each experiment the feed consumption was equalized for each two comparative groups.

The data seem to furnish strong support for several suppositions. First, there is a marked seasonal difference in the susceptibility of chicks to rickets. A further discussion of this difference has been given by Lachat ('34). Second, a greater rachitogenic property of oats is demonstrable when the degree of rickets is mild, and (or) the susceptibility less. Third, when the rachitic condition is extreme a further concentration of the factors causing it does not produce a still further lowering of the bone ash. Fourth, the degree of rickets produced in the first and fourth experiment with yellow corn being the same and also like that in the test for oat concentrate C reported above, which was conducted simultaneously with the fourth experiment, this concentrate evidently did possess rachitogenic substances.

#### CONCLUSIONS<sup>4</sup>

1. Hydrochloric acid extracts of oats, when purified and freed from excessive amounts of NaCl, may exhibit rachitogenic properties when fed to rats in a mildly rachitogenic ration, but not when the ration is severely rachitogenic.

<sup>4</sup>The conclusions are taken essentially verbatim from Doctor Lachat's thesis.

2. Rolled oats and yellow corn are rachitogenic to both rats and chicks, especially to the latter, and rolled oats appears to be more rachitogenic when the rations are otherwise only mildly rickets producing or the susceptibility of the animals is low.

3. Divergent results obtained by others in the study of the relative rachitogenic properties of oats and other cereals probably may be explained by lack of control of the severity of the rickets produced by the ration with which the cereal rations have been compared.

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# METABOLISM OF WOMEN DURING THE REPRODUCTIVE CYCLE

## VI. A CASE STUDY OF THE CONTINUOUS NITROGEN UTILIZATION OF A MULTIPARA DURING PREGNANCY, PARTURITION, PUERPERIUM AND LACTATION

HELEN A. HUNSCHER, FRANCES COPE HUMMELL, BETTY NIMS  
ERICKSON AND ICIE G. MACY

*Research Laboratory of the Children's Fund of Michigan, Detroit*

### THREE FIGURES

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### INTRODUCTION

Many types of observations have been made in this laboratory during the past 12 years that have shown significant individual differences in metabolic processes of healthy, non-pregnant, pregnant, lactating and post-lactating women even when the subjects were studied under apparently similar dietary, environmental and experimental conditions. These individual fluctuations have been reflected not only in the metabolic balances of pregnancy and lactation but in the quantity of milk flow, the chemical composition and biological properties of the milk, the constituents of the blood, the voluntary choice and quantity of food consumed, the physical state and emotional stability of the individual. In view of these disparate findings we have emphasized on numerous occasions that single metabolic balance periods of 4 or even 10 days' duration, conducted at specific intervals during the progression of the reproductive cycle, have unquestionable usefulness in approximating food utilization and evaluating metabolic adjustments, providing a suffi-

ciently large number of observations are obtained. Due, however, to a multiplicity of determinate factors that do not lend themselves to control, data accumulated over brief intervals should not always be considered as representing physiological constants peculiar to a given stage of reproduction of women in general (Macy, Hunscher, Nims and McCosh, '30; Hunscher, Donelson, Nims, Kenyon and Macy, '33). In view of the above facts it is expedient then to extend, not only the short balance to a longer period of time, but to determine uninterruptedly the metabolic responses of the same mother during the development of the fetus and the physiological preparation of her own body for lactation, and thereafter, to extend these observations into parturition, puerperium and lactation thereby learning more specifically where the stresses and strains of child-bearing and child-rearing lay. Such a continuous evaluation, even in the case of one healthy woman during the reproductive cycle as a whole will render service since, to our knowledge, no such information exists at the present time.

Hoffström's ('10) classical observation showed, in a normal woman studied continuously from the seventeenth week of pregnancy to term, that the maternal tissues were enriched during pregnancy beyond the needs of the fetus thus resulting in an appreciable accumulation of 'rest material.' Subsequently, the metabolic balances made in this laboratory (Hunscher, Donelson, Nims, Kenyon and Macy, '33) on the same individuals at intervals during both pregnancy and lactation in multiparae indicated appreciable storage in pregnancy on the one hand, but on the other, losses in some cases from the maternal body in lactation. In view of the urgent want for knowledge on the same individual uninterruptedly in gestation and the subsequent lactation, it is significant to both infant and maternal welfare to measure the total physiological effect of reproduction upon the maternal tissues and to determine whether the losses occurring in parturition, puerperium and early lactation exceed the storage acquired during pregnancy, and thereby cause an actual and conse-

quential drain upon the maternal body, or whether the maternal body is left enriched. As a contribution to the pertinent need for more detailed information on the chemical exchanges<sup>1</sup> taking place during the reproductive cycle of a healthy woman, the present paper records continuous metabolic balance data on the progressive nitrogen utilization of a multipara during 21 weeks of gestation through delivery and puerperium to the eighth week postpartum, a total observation period of approximately 8 months; for comparative purposes data collected from balances made at intervals during the two preceding reproductive cycles are included. For the sake of brevity only references that are pertinent to a succinct presentation of the immediate data on human subjects are included since they have been adequately reviewed in recent literature.

#### EXPERIMENTAL

*Subject.* An American woman (L.R.)<sup>2</sup> of Scotch-Irish descent, who had already been under the constant observation of this laboratory staff for more than 5 years, including the duration of two uneventful and successful lactations and one pregnancy, again made herself available for continuous metabolic observations during the early part of her fourth reproductive cycle. She enjoyed buoyant health at all times. Frequent medical examinations<sup>3</sup> over a period of years showed her to have an excellent record and to be free from metabolic disturbances. Her normal weight approximated 135 pounds, which is satisfactory for a woman in her thirties with a height of 68.5 inches. Table 1 giving her chronological reproductive history shows the frequency of pregnancies, her condition during parturition and the habitual long and heavy milk flow. Except for confinement, the subject remained in her own home and performed the usual household duties in caring for her

<sup>1</sup> The acid-base mineral balances are to be reported in a subsequent paper.

<sup>2</sup> Referred to in previous publications on milk flow, food consumption, chemical and biological properties of milk and isolated metabolic studies as subject L.R. or case VII.

<sup>3</sup> Grateful acknowledgment is made to Dr. A. S. Guimaraes for his interest in the study, his care of the patient and his untiring cooperation.

home and family. The three former children are healthy, mentally alert with intelligent quotients considerably above average, and emotionally equipoised from birth.

*Dietary.* Since the subject over a period of years had been accustomed to voluntarily selecting, both quantitatively and

TABLE 1  
*Chronological reproductive history (subject L.R. or VII)*

NUMBER OF REPRODUCTIVE CYCLES	1	2	3	4
Pregnancy				
Age, years	30	32	33	37
Last menstrual period	Feb. 27, 1924	Feb. 15, 1926	Nov. 18, 1927	Sept. 25, 1931
Duration, days	252	280	301	280
Albumin	Negative	Negative	Negative	Negative
State of health	Good	Excellent	Excellent	Excellent
Gain in weight, pounds	25	28	26½	26
Parturition				
Date	Nov. 5, 1924	Nov. 20, 1926	Sept. 14, 1928	July 4, 1932
Type of delivery	Normal	Normal	Normal	Normal
Duration of labor, hours	5	6½	6	4½
Condition of subject	Good	Excellent	Excellent	Excellent
Hemorrhage	Slight	Little	Little	Slight
Birth weight of infant, pounds	4.25	8.25	9.25	8.75
Lactation				
Average daily milk secretion, ounces	80	67	78	50
Duration, weeks	60	50	62	40
Voluntary cessation	Jan. 3, 1925	Nov. 10, 1927	Dec. 5, 1929	April 10, 1933
Post lactation, months	.....	1½	1 week	21½

qualitatively, a well-balanced diet, her usual choice of food was not interfered with during the present study. In addition to the usual fruits, vegetables, cereals and meat she took daily 2 quarts of milk in each of which were incorporated 400 U.S.P. units of vitamin D in the form of a cod liver oil concentrate (Vitex)<sup>4</sup> thus insuring a total food intake that was

<sup>4</sup>The authors and subject are indebted to Dr. Bion East of the Vitex Laboratories Inc., Harrison, N. J., for furnishing the fortified cow's milk during this study.

abundant in all the known essential nutritive principles. In this food consumption her caloric intake averaged 3378 cal. (2524 to 4019) in pregnancy and 3400 cal. (2490 to 4324) in lactation.

*General procedure.* Successive metabolic balance periods each of 5 days' duration were begun in the reproductive cycle at approximately the one hundred and thirty-fifth day of gestation and carried thereafter continuously. The food was weighed before consumption and at the same time one-half by weight of each serving was placed in a container for chemical analyses. The salt and sugar were served separately and accounted for by period. The feces were marked off by carmine and nitrogen determinations were made on a composite sulfuric acid digest of the fresh material to avoid possible losses during drying (Hawk and Bergeim, '31) The daily urine specimens were preserved with toluol and kept in a refrigerator, and at the end of each metabolic balance period aliquots of the daily collections were made up into a composite for chemical analyses. All nitrogen determinations on food, urine and feces were made by the macro Kjeldahl method except for the urine during lactation when the gasometric method of Van Slyke ('26-'27) was used.

During delivery in the hospital<sup>5</sup> all specimens of maternal body losses were collected including those from the linens from which the blood, amniotic fluid, and other excretions were leached out with distilled water and the extract analysed for nitrogen. By the analyses of the irrigation water, blood, placenta and amniotic fluid it was possible to make a reasonably accurate account of the maternal losses during parturition over and beyond that represented by the fetus.

Throughout the puerperium the absorbent pads containing the blood loss and lochia were analysed. Beginning with the third day postpartum as soon as the mammary secretion

<sup>5</sup> The medical, nursing and dietetic staffs of the Florence Crittenton Hospital gave generous cooperation during the 10 days' confinement period. Graduate nursing service was maintained by the laboratory for the continuous care of the patient, collecting samples and making necessary records.

started the milk was completely emptied from both breasts at 4-hour intervals and aliquot samples were collected for the chemical determinations. During the weeks of lactation the breast milk of each complete day's production was obtained by manual expression at regular intervals each day and reserved as part of the metabolic specimens and the remaining milk was given to the baby by bottle and any surplus was sold to the Detroit Mother's Milk Bureau. It is important to note that this mother by means of her hereditary or environmental endowment was capable of providing a generous quantity of breast milk sufficient to promote excellent growth in her former infants and at the same time furnished additional quantities for a variety of other purposes. Moreover, she manually expressed her breast milk during each reproductive cycle and was, therefore, accustomed to lactating in the absence of the suckling influence with no resultant emotional upset. The frequent and complete removal of milk from the mammary glands stimulated a maximum and uniform milk flow, the reputable quality of which was judged by numerous chemical and biological tests.

During the same interval she became used to weighing her own food and accurately collecting the metabolic specimens, and being an intelligent woman with some scientific training she enjoyed having a part in the studies, because she considered them not only of scientific value to others but she herself derived a considerable degree of personal satisfaction from participating in them.

#### RESULTS AND DISCUSSION

Tables 2 and 3 give a summary of the data accumulated on the continuous nitrogen retention of one woman in her fourth reproductive cycle. The quantity of nitrogen taken into the body over a 7-month period including the final 145 days of pregnancy, parturition and 53 days after delivery during heavy milk flow, the paths of excretion in the urine, feces and breast milk and the maternal gain or loss are recorded. In addition, the data are supplemented by results from indi-

TABLE 2  
*Continuous daily nitrogen balances during pregnancy of subject L.R.*

DAYS OF PREGNANCY	WEIGHT OF SUBJECT	NITROGEN INTAKE	NITROGEN OUTGO		NITROGEN BALANCE		
			Urine	Feces	Net	Fetus and adnexa <sup>1</sup>	Maternal
	<i>kilo</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
135-141	71.2	19.55	13.84	1.96	+ 3.75	0.13	3.62
142-148	71.7	19.21	14.66	2.22	+ 2.33		2.20
149-155	72.0	17.76	13.38	1.94	+ 2.44		2.31
156-162	72.3	18.17	13.46	2.06	+ 2.65	0.23	2.42
163-167	72.6	18.83	14.09	2.30	+ 2.44		2.21
168-170	73.0	15.80	13.06	1.80	+ 0.94		0.71
171-175	73.3	21.42	12.73	2.09	+ 6.60		6.37
176-180	73.8	19.02	15.40	1.68	+ 1.94		1.71
181-185	74.3	19.86	15.39	2.00	+ 2.47	0.39	2.08
186-190	74.7	15.51	12.86	1.73	+ 0.92		0.53
191-195	75.1	22.33	14.69	3.13	+ 4.51		4.12
196-200	75.3	18.34	13.26	1.84	+ 3.24		2.85
201-205	75.6	16.95	12.28	1.81	+ 2.86		2.47
206-210	75.9	20.06	13.57	1.89	+ 4.60	0.22	4.38
211-215	76.2	21.25	15.44	1.59	+ 4.22		4.00
216-220	76.5	20.12	14.97	1.80	+ 3.35		3.13
221-225	76.8	21.10	13.61	2.05	+ 5.44		5.22
226-230	77.0	19.12	14.02	2.01	+ 3.09		2.87
231-235	77.3	18.21	14.62	1.95	+ 1.64	0.71	0.93
236-240	77.5	19.26	15.07	1.96	+ 2.23		1.52
241-245	77.7	18.45	13.80	1.58	+ 3.07		2.36
246-250	77.9	19.52	15.23	2.42	+ 1.87		1.16
251-255	78.0	18.92	14.30	1.70	+ 2.92		2.21
256-260	78.1	18.80	14.15	1.67	+ 2.98	1.18	1.80
261-265	78.2	18.34	13.56	1.93	+ 2.85		1.67
266-270	78.3	17.40	12.26	1.94	+ 3.20		2.02
271-275	78.4	18.76	14.02	1.44	+ 3.30		2.12
276-280	78.5	19.45	13.76	2.38	+ 3.31		2.13
Mean		19.01			+ 3.06		2.60
Total gain	7.3				446.2		
Day of delivery		18.05 food	5.84 urine	0.40 feces			
			25.75 blood				
			20.11 placenta				
			20.28 blood from placenta				
			0.08 amniotic fluid				
			0.24 vomitus				
			72.70 total outgo			— 54.65 balance	

<sup>1</sup> The average figures were calculated from a previous review (6).



vidual short time nitrogen balance periods at intervals in the second lactation and during the third cycle including post-lactation (figs. 1 and 2).

*Pregnancy.* The data on nitrogen retention during pregnancy are given in table 2 which shows the intake, paths of outgo and the net and maternal balance. The latter was

### NITROGEN RETENTION IN A WOMAN DURING THREE SUCCESSIVE REPRODUCTIVE CYCLES

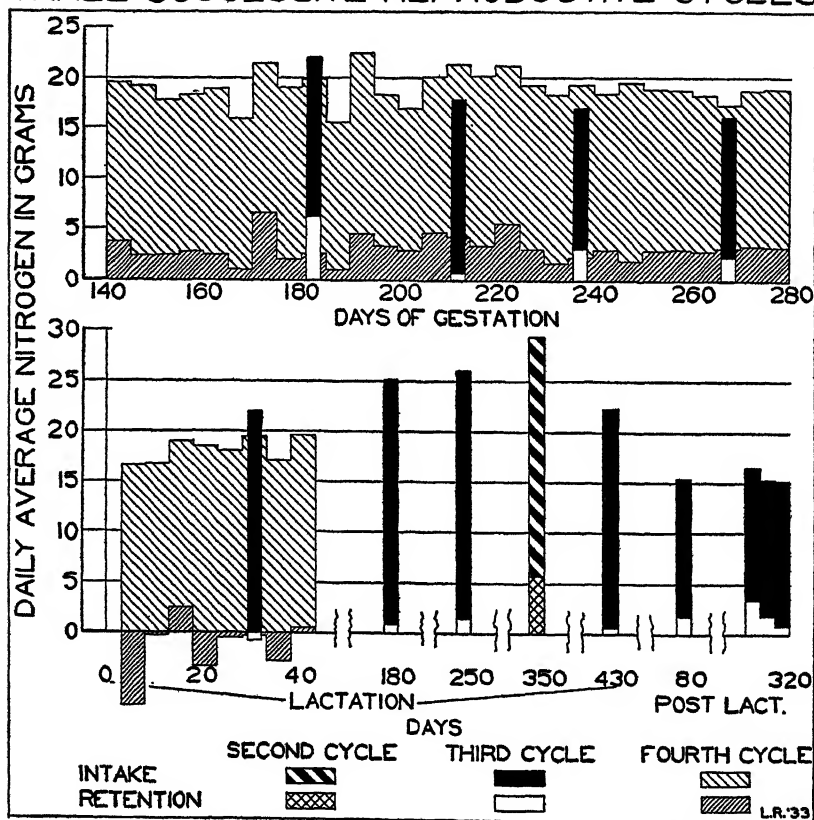


Fig. 1 Continuous nitrogen intake and retention of a woman during the last 145 days of pregnancy through puerperium to the eighth week postpartum in her fourth reproductive cycle upon which are superimposed isolated metabolic balances that were made on the same subject during the two preceding cycles. The average daily grams of nitrogen are plotted on the vertical scale and the days in the periods of the reproductive cycles are given on the horizontal scale.

calculated by the difference between intake and the sum of the excretions and fetal needs. With an average ingestion of 19 gm. (15.8 to 22.3 gm.) of nitrogen from proteins of excellent quality as derived from 2 quarts of milk daily and a well-balanced diet there was a resultant mean net retention of 3.1 gm. (0.92 to 6.6 gm.) per day, thus representing a storage of 16 per cent of the nitrogen intake during the last 145 days of the fourth pregnancy. The ability of the present subject to retain an abundance of nitrogen was further evidenced by the fact that she was able to store an average of 2.6 gm. of nitrogen daily beyond the calculated daily acqui-

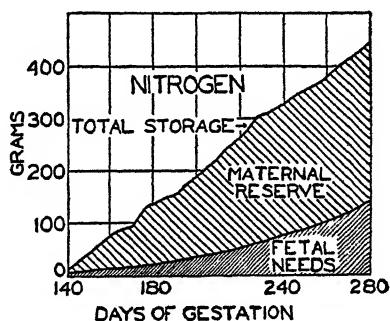


Fig. 2 Determined maternal and estimated fetal storage of nitrogen during the final 145 days of gestation. The maternal retention greatly exceeds the progressive needs of the fetus and its adnexa.

tion of the fetus which weighed 3.7 kg. at term and its adnexa (Macy and Hunscher, '34). It is significant to note that these continuous observations are in agreement with the findings from four metabolic nitrogen balances of 5 days' duration each that were made at monthly intervals in the last half of the third pregnancy when a diet similarly abundant in nitrogen and calories resulted in an average daily net storage of 2.89 gm. (fig. 1).

In the present study the highest retention occurred with the greatest nitrogen intake, but this did not always follow. Two-thirds of the net nitrogen balances were concentrated between 2 and 3.5 gm. retention per day with their intakes varying from

17 to 20 gm. In a previous report (Hunscher, Donelson, Nims, Kenyon and Macy, '33) where the data from the literature on nitrogen requirements of pregnancy and lactation have been assembled, it was shown that of the data from 954 daily balances 48 per cent fell within the range of 1 to 3 gm. of nitrogen retention, figure 3. Furthermore, 80 per cent of the intakes extended from 10 to 18 gm. per day while the retentions came within the range of 1.5 to 2.7 gm. The average daily retention throughout pregnancy from this compilation was 2.28 gm. while for the present subject under continuous

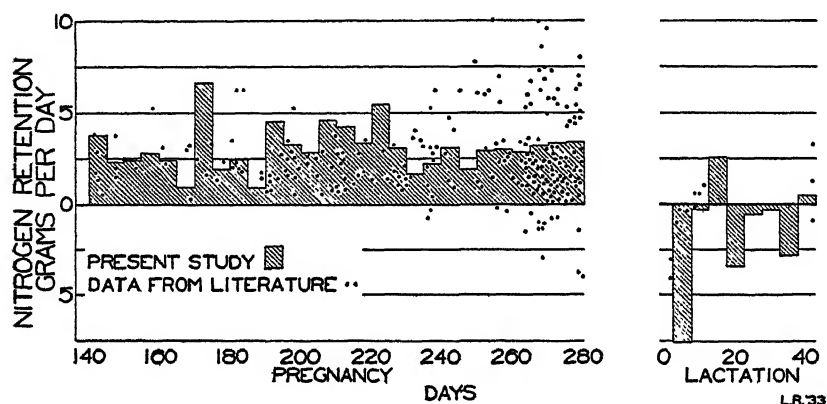


Fig. 3 Nitrogen retentions in grams per day of the present continuous study in pregnancy and lactation upon which are superimposed the scattered nitrogen balances upon women that are available in the literature.

observation it amounted to 3.1 gm. On comparison of the two sets of data, it seems that, in general, the richer the diet in protein and that of good biological values such as from milk and its products, the greater was the persistent storage of nitrogen during pregnancy. These indications corroborate the conclusions of Coons and co-workers (Coons and Blunt, '30; Coons and Marshall, '34; Coons, '35). The physiological advantage of a generous store of nitrogen throughout gestation seems evident, but additional clinical demonstrations are needed.

In pregnancy the amount of tissue building constituents stored in the gravid individual depends upon, 1) the usual

wear and tear of maintenance requirements of the adult individual, 2) the developing fetus and its adnexa, 3) the preparation of the maternal body for parturition and lactation throughout a building up of an excess body store including the hypertrophy of many maternal organs notably of the breasts and uterus, 4) the filling in of the nutritive reserves that perchance were existent through a previous state of undernutrition, and in some cases, 5) upon the growth of the maternal body itself, and 6) the quality and quantity of food.

The total accumulation of nitrogen in the maternal body during the last 145 days of prenatal development amounted to 446 gm. This storage was greater than that reported by Hoffström ('10) from observations begun 2 weeks prior in the term of pregnancy and made continuously to delivery. He found an average daily storage of 1.84 gm. resulting in a total accretion of 310 gm. at term from which were taken 101.5 gm. for calculated fetal needs, leaving 208.5 gm. of 'rest material.' Slemons ('04) and Wilson ('16) found similar amounts. In this study the calculated fetal and maternal needs including estimated utilization of nitrogen in the mammary glands and uterus were estimated to be 146 gm. leaving a remainder of 300 gm. of 'rest material' acquired during the last half of pregnancy by the subject under investigation, figure 2. This abundant storage of maternal nitrogen may be accounted for either by an unusually generous food consumption, or a somewhat depleted maternal body produced by frequent pregnancies followed by long and heavy lactation, and finally, a physiological preparation for an oncoming heavy milk production period. Subject L.R. did not experience any nausea in early pregnancy and it is quite possible that her total nitrogen storage accompanying the gestation period was considerable during the early part of the term before chemical studies were begun. The total gain in body weight during the last 145 days of pregnancy amounted to only 7.3 kg. but the retention of 446 gm. of nitrogen in terms of muscle weight would approximate 14 kg. calculating muscle as 20 per cent protein. It is evident that much remains to be learned of the

fate of the nitrogen retained during pregnancy in the maternal preparation for parturition and the physiological needs of lactation as well as the factors governing the composition and shifts in body weight changes and in addition, the distribution between the maternal body and the fetus on the one hand and breast milk on the other.

*Parturition.* Parturition and labor made the first toll on the maternal reserve accumulated during pregnancy. The total chemically analyzed loss through urine, feces, placenta, blood, vomitus and amniotic fluid during delivery amounted to 5.84, 0.40, 20.11, 46.03, 0.24 and 0.08 gm. of nitrogen, respectively, yielding a total known loss of 72.70 gm. or 54.65 gm. of nitrogen in excess of the food ingested (table 2). In addition, the infant weighed 3.7 kilos at birth and if its body composition were equivalent to the average figure obtained through the analyses of nine fetuses at term (Macy and Hunscher, '34) it contained approximately 58.6 gm. of nitrogen. The total outgo of nitrogen from the maternal body including the urine, feces, placenta, blood and amniotic fluid and the full term fetus beyond the food consumed amounted to approximately 113 gm. on the day of delivery which left a maternal capital of 333 gm. of nitrogen for future dissipation during puerperium and lactation.

*Puerperium.* The metabolic nitrogen balance data for the 9 days' postpartum spent in the hospital are shown in table 3. Although the daily food intake was generous with an average of 16.4 gm. (14.4 to 18.5 gm.) of nitrogen, there was a total loss during the 9 days of 44.6 gm. varying from + 2 to - 7.6 gm. of nitrogen per day. This loss during puerperium is proportionate to the results obtained by other investigators (Slemons, '04; Wilson, '16; Mellanby, '12-'13; Harding and Montgomery, '27; and Shaffer, '08-'09). Whether this large nitrogen loss and the creatinuria of puerperium are referable primarily to the involuting uterus or in part to the physiological readjustment from the prenatal state to the inception of lactation or to both is still an open question.<sup>6, 7</sup>

<sup>6</sup> An intensive metabolic study will soon be published on a young primipara.

<sup>7</sup> A detailed consideration of the nitrogen partition of the urine and milk will be reported later.

Slemons ('04) reported that a gravid uterus contained approximately 39 gm. of nitrogen. From the maternal 'rest nitrogen' of 333 gm. that were left after the day of delivery in the present study, the first 9 days of the puerperium made an additional toll which included the inestimable losses from

TABLE 3

*Continuous daily nitrogen balances during puerperium and lactation of subject L.B.*

DAYS OF PUERPERIUM	WEIGHT OF SUBJECT	NITROGEN INTAKE	NITROGEN OUTGO					NITROGEN BALANCE
			Urine	Feces	Pads	Breast milk	Irriga- tion	
1		15.67	13.93	2.75	0.87	....	0.08	- 1.96
2		14.38	12.70	....	7.95 <sup>1</sup>	....	0.29	- 6.56
3		16.56	12.46	....	0.19	1.67	0.14	+ 2.10
4		13.52	13.11	3.95	0.53	5.17	0.38	- 4.62
5		16.10	11.32	4.46	0.24	5.17	0.17	- 5.26
6		17.74	16.76	1.39	0.22	5.87	0.50	- 7.00
7		17.20	16.26	1.39	0.17	6.14	0.46	- 7.22
8		15.77	14.65	1.39	0.04	6.88	0.38	- 7.57
9		15.19	13.71	1.39	0.05	6.50	0.09	- 6.55
Total		147.13	124.90	16.72	10.26	37.40	2.49	- 44.64
Mean		16.35	13.88	1.86	1.14	4.16	0.28	- 4.96
DAYS OF POST PARTUM								
10-17	65.9	17.56	13.85	1.41	0.51	4.21		- 2.32
18-22		16.75	12.24	1.49	0.51	2.64		- 0.13
23-27		19.00	12.10	1.58	0.24	2.52		+ 2.55
28-32		18.55	13.37	3.30	0.18	5.10		- 3.40
33-37	66.0	18.06	11.48	1.79	0.11	5.23		- 0.55
38-42		19.52	12.82	2.18	....	4.65		- 0.13
43-47		17.07	13.02	1.82	....	5.01		- 2.78
48-53		19.60	12.59	2.62	....	3.90		+ 0.49
Total		802.83	561.49	87.80	9.28	182.83		- 38.31
Mean		18.36	12.76	2.00	0.21	4.16		- 0.87

<sup>1</sup> Clot of blood lost.

the involuting uterus leaving 288.4 gm. of nitrogen for use during the succeeding days of lactation and postlactation. Many more cases will have to be studied intensively and continuously before it is possible to determine the actual nitrogen requirements of the average reproductive cycle including the sum total of the physiological gains and losses.

*Lactation.* During the 43 days of lactation following the period of puerperium of 9 days there was a maternal loss of 38.3 gm. of nitrogen or an average of 0.87 gm. daily as shown in table 3 and figure 3. The body weight of L.R. at the fifteenth day postpartum was 65.9 kilos, a weight approximating her usual non-gravid state. Altogether then the puerperium and 6 weeks of lactation decreased the 'rest nitrogen' from 333 to 250 gm. The inability to extend the continuous metabolic studies further into lactation made it impossible to determine when this maternal body reached an equilibrium and whether it was actually left enriched at the termination of the reproductive cycle or whether the 'reserve material' of gestation was completely dissipated. Data previously reported (Hunscher, Donelson, Nims, Kenyon and Macy, '33) on the response of the present woman to analagous reproductive metabolic states when she was consuming similar nitrogen intakes at the fiftieth week of the second lactation showed that she was storing 5.7 gm. of nitrogen per day and, at the seventh, twenty-sixth, thirty-sixth and sixty-second weeks of the third lactation the daily nitrogen balances were  $-0.84$ ,  $+0.87$ ,  $+1.45$  and  $+0.61$  gm., respectively. Furthermore, at the eleventh and forty-sixth weeks postlactation of the latter cycle the balances were positive with a lowered consumption of nitrogen.

From the continuous metabolic studies of this case during a large part of a reproductive cycle and at intervals in two preceding cycles it is evident that losses occur especially during early lactation in some multiparae<sup>6\*</sup> but the maternal body can attain equilibrium and even store nitrogen during heavy milk flow after the twenty-sixth week. This observation was borne out in two other women with high milk production records (Hunscher, Donelson, Nims, Kenyon and Macy, '33). If the large losses of nitrogen in early lactation are due in part to involutional processes<sup>6,7\*</sup> of the maternal organs (Harding and Montgomery, '27) and are therefore physiologic, and, are provided for by the 'rest material' accumulated

<sup>\*\*</sup> and <sup>\*,7\*</sup> See footnotes 6 and 7 on page 590.

in pregnancy, it seems important to search out methods of providing the mother with an ample reserve to aid against vital losses at the time of delivery and during the period when milk flow is being established. Whether excessive losses can be reduced by larger intakes of nitrogen (i.e., more than 18.4 gm. of nitrogen or 115 gm. of protein) or through other dietary and hygienic means remains to be investigated by further continuous balance studies and verified in clinical practice.

Only a few data are available on the nutritive needs for human milk secretion (Hoobler, '17; Courtney, '23; Adair, '25; Kleiner, Tritsch and Graves, '28). Since the subject of this investigation secreted a large quantity of breast milk which was completely expressed manually at regular intervals in each 24-hour period and the chemical composition determined during the entire study it is possible to assess the protein requirement under her specific metabolic demands. The maternal maintenance requirement, the loss in the milk secreted and the incalculable metabolic cost for the mammary gland activity in the synthesis of a milk of known quantity and quality constitute the protein need. L.R. secreted an average of 26.5 gm. of protein in her milk from the tenth to the fifty-third day of lactation. If her protein maintenance requirement did not exceed the usual 70 gm. allowance for the adult and if the requirement for milk secretion equals the outgo of protein in the milk then her calculable requirement amounted to 96.5 gm. daily. She used, however, an average of 120 gm. of protein daily as shown by the loss of 5.4 gm. over and beyond the 115 gm. accounted for in her food intake when as little as 26.5 gm. of protein occurred in breast milk. It seems then that nitrogen was not only used or dissipated in the physiological readjustment to the establishment and maintenance of milk flow but in the actual milk synthesis. In addition, it is possible that in some cases other factors may be operating, such as an undernourished state due to either an inadequate food supply or insufficient utilization of the food taken prior to conception, an actual pathological state during



gestation, or an inability of the body to retain the 'rest material' of pregnancy on the one hand, or on the other hand, a drain placed upon the maternal body through frequent and intensive reproductive cycles as may be true in the present subject. These considerations illustrate some of the difficulties involved in the computation of actual and general dietary requirements for reproduction and point to the need for more skillful dietary and clinical means of safeguarding the maternal tissues from undue deprivation.

The continuous metabolic balance studies reported herein show variations in retentions which, perforce, are inherent in data secured from isolated intermittent observations. The continuous findings are more valid since they show not only the general trend of metabolism during the different phases of the reproductive cycle but also show the great fluctuations that may occur in short periods under apparently similar experimental conditions. These results are in accord with the previous data on women undergoing the reproductive cycle in which we have shown that "there is a marked variation in the storage of nitrogen in the same individual despite the maintenance of a fairly constant consumption of protein, where consecutive balances are determined. Such evidence indicates that a single balance does not represent a physiological constant of metabolism and consequently it should not be used too freely in generalizations" (Hunscher, Donelson, Nims, Kenyon and Macy, '33). Likewise, the present data are singularly substantiated through observations obtained in successive nitrogen balances in childhood.<sup>8</sup>

From the data accumulated in this laboratory over a period of years it appears that the most satisfactory means of assessing the physiologic load of maternity and of determining the nutritive state of women at the termination of the reproductive cycle is by observing continuously the retention or loss of the various elements throughout each successive phase

<sup>8</sup> An elaborate study on successive metabolic balances in childhood is to be published at an early date (Hunscher, Cope, Noll, Macy, Cooley, Penberthy and Armstrong, '32; Hunscher, Cope, Noll and Macy, '33).

of pregnancy, delivery, puerperium and lactation with a consequent accounting of the total demands made upon the maternal tissues throughout the entire cycle. It has been a rare and unprecedented opportunity to be able to collect from the same individual during the major portion of her years of child-bearing and child-rearing metabolic data from three successive and physiologically similar reproductive cycles covering almost 8 years and to be able to supplement them with observations on women satisfactorily carrying similar metabolic loads. In addition, it is a matter of practical health interest to learn the consequential effect of child-bearing upon the well-being of the mother and her child under various types of normal conditions and abnormal states that frequently arise in pregnancy, parturition, puerperium and lactation.

It is through the accumulation of many varieties of chemical exchange data on numerous women beginning, if possible, before conception and following throughout pregnancy, parturition, puerperium and lactation and even beyond, until the maternal body reaches a state of equilibrium that knowledge for the solution of so many urgent problems will be found. With a fuller knowledge of the maternal metabolic processes, physicians will be better prepared to guide mothers to enjoy buoyant health and at the same time to bear nutritionally stable children.

#### SUMMARY

A case study extending over an 8-year period of child-bearing and child-rearing has been made on a woman when she and her children were known to enjoy buoyant health.

Uninterrupted nitrogen metabolic responses during the last half of fetal development and the physiological preparation of the maternal body for lactation and the extension of these observations into parturition, puerperium and 8 weeks of lactation showed where some of the stresses and strains of maternity lay. The results confirm previous considerations derived from intermittent balances in two former reproductive cycles in the same woman.

During the last 145 days in gestation there was an average net storage of 3.1 gm. and a maternal retention of 2.6 gm. of nitrogen daily resulting in a total observed accumulation of 446 gm. at term.

On the day of delivery the chemically determined maternal loss in blood, placenta, amniotic fluid and vomitus amounted to 46.0, 20.1, 0.08, 0.24 gm. of nitrogen, respectively; the total loss from the body beyond the food consumed amounted to 54.6 gm. of nitrogen in addition to that contained in the fetus.

The nine daily balances during the lying-in period showed an average daily loss of 5 gm. of nitrogen.

From the tenth to the fifty-third day of lactation there was an average daily loss of 0.87 gm. of nitrogen.

By the fifty-third day of milk flow the gestatory reserve nitrogen had been reduced by delivery, puerperium and lactation losses of 54.6, 44.6 and 38.3, respectively leaving a total of 310 gm. of nitrogen stored only in the last half of pregnancy. When the approximate fetal content of 58.6 gm. of nitrogen is deducted from the final maternal reserve, the accountable losses of the reproductive cycle by the fifty-third day of lactation had left a maternal reserve of 250 gm. of nitrogen for future dissipation or enrichment of the maternal body at termination of the reproductive cycle.

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## SELENIUM IN PROTEINS FROM TOXIC FOODSTUFFS <sup>1</sup>

### IV. THE EFFECT OF FEEDING TOXIC PROTEINS, TOXIC PROTEIN HYDROLYSATES, AND TOXIC PROTEIN HYDROLYSATES FROM WHICH THE SELENIUM HAS BEEN REMOVED

KURT W. FRANKE AND E. PAGE PAINTER

*Department of Experiment Station Chemistry, South Dakota State College,  
Brookings*

TWO FIGURES

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It was stated in an earlier paper (Franke and Painter, '35 a) that the only difference known between 'normal' and toxic foodstuffs is the presence of selenium in the latter. Vanadium may contribute to the toxicity of some of the grains as Byers ('34) found this element in one sample. Beath, Eppson and Gilbert ('35) have reported that plants may take up molybdenum in amounts which will produce toxic symptoms when fed to livestock. They state that molybdenum is less toxic than selenium.

Franke ('34 b) reported that the protein carried the toxicant in toxic foodstuffs and Robinson ('33) and Franke and Painter ('35 a) found the selenium in the protein. The latter workers also reported that the selenium was in organic combination in the protein and was in solution after the protein was hydrolyzed. Painter and Franke ('35 b) have developed

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a procedure whereby the selenium compounds can be removed from the hydrolysate.

Several papers have appeared from this laboratory on the effects of toxic foodstuffs on the animal body. However, no report has appeared on the toxicity of the derived products of toxic proteins.

In view of the above facts, it seemed advisable to determine by growth studies on the albino rat the relative toxicity of the grain, the protein, and the protein hydrolysate, and to determine if the removal of selenium would render the protein hydrolysate non-toxic.

#### EXPERIMENTAL

In order to determine the effect on animals of the chemical treatment used to separate the selenium compounds, the following feeding trials (series 101) were carried out.

Group A was fed a diet containing toxic wheat.

Group B was fed a diet containing toxic proteins.

Group C was fed a diet containing the hydrolysate from toxic proteins.

Group D was fed a diet containing the hydrolysate from toxic protein from which the selenium had been removed.

It should be emphasized that in these feeding trials strict series of controls were used. In all feeding trials a fraction was fed from a 'normal' wheat (laboratory no. 648) which had received the same treatment as the experimental. These will be referred to as control groups A, B, C and D.

The protein from toxic wheat (laboratory no. 582) fed to group B was prepared by the same procedure as that used in previous investigations (Franke and Painter, '35 a).

The following method was used to prepare the hydrolysates for animal feeding. The hydrolysis of proteins has been described (Painter and Franke, '35 a). The insoluble humin was filtered off and the sulfuric acid removed in the manner already described for preparing the hydrolysates prior to the separation procedures (Painter and Franke, '35 b). Care had to be taken to ensure that there was no barium in the

hydrolysate. It was found that excess sulfuric acid had to be present before all the barium was removed. The pH of the hydrolysate was acid (between 4 and 5), but indicators could not be depended upon. Instead dilute sulfuric acid was added until no more barium sulfate precipitated, then several cubic centimeters of very dilute acid were added. The solution was concentrated to a thick syrup by vacuum distillation at not over 60°C. The syrup was removed from the distillation flasks and placed in evaporating dishes. At this time sodium hydroxide was added until the end point of methyl red was reached. The neutral hydrolysates were then placed on a steam bath to remove most of the water, then finally dried in a vacuum oven at 60°C. The amino acids were then ground in a mortar, and kept in a desiccator until incorporated into diets and fed to group C.

#### *Preparation of the selenium free hydrolysates*

The method of precipitating the selenium from toxic protein hydrolysates has been described (Painter and Franke, '35 b), but the essential features of the precipitation will be restated.

The neutral hydrolysate (free from sulfates and barium) was diluted to a volume of 30 cc. for every gram of protein hydrolyzed. Solid barium carbonate was added in excess, then for every 30 cc. of hydrolysate 20 cc. of saturated mercuric chloride was added while the solution was stirred. The solution was allowed to set at room temperature, with frequent stirring, for 1 hour. The flaky mercury precipitate and undissolved barium carbonate was filtered off, using a Buchner funnel, and the precipitate washed several times with water. A precipitate may form in the clear solution after the mercuric precipitate of amino acids and excess barium carbonate have been filtered off if it is allowed to stand, but this may be disregarded. The mercury in the filtrate was removed with hydrogen sulfide and the barium with dilute sulfuric acid. The mercury, barium and sulfate-free filtrate was evaporated to near dryness several times in vacuum to remove as much chlorine as possible. The filtrate was then made up to a



suitable volume and the remaining chlorine removed by the addition of boiling hot suspensions of silver oxide. This was added until the pH reached 4. The silver chloride and excess silver oxide was then filtered off and washed several times with hot water. The small amount of silver in the solution was removed as the sulfide by the addition of hydrogen sulfide and the volume of the solution reduced by vacuum distillation until some amino acids began to crystallize out. The solution was then tested with a few cubic centimeters of dilute sulfuric acid to ensure complete removal of barium. If it was free from all toxic metals, dilute sodium hydroxide was added and the pH adjusted to that described for the hydrolysates. This amino acid fraction was then dried in the manner already described. Each one of these filtrates was tested for the presence of selenium before it was incorporated into diets and fed to group D. The same procedure was carried out using a hydrolysate of control protein which served as a control group (D). The usual practice was to hydrolyze 300 gm. of protein at one time and divide the neutral hydrolysate, taking half for the hydrolysate diet and carrying the other half through the precipitation procedure.

#### *Preparation of the diets*

The diets were prepared so that their nitrogen contents were equal. It has been stated (Franke, '34 b; Franke and Painter, '35 a) that the toxicant was carried in the protein of toxic foodstuffs so protein nitrogen was used as the basis for the preparation of the diets.

The wheat diets were the same as those used by Franke and Potter ('34). The following table gives the diets used in each of the eight groups:

Toxic and control groups	SERIES 101 DIETS			
	A	B	C	D
Component in per cent				
Ground whole wheat	82			
Commercial casein	10	10	10	10
Cod liver oil	2	2	2	2
Dehydrated yeast (Northwestern)	2	2	2	2
McCollum's salt mixture no. 185	1	3	2	2
Lard	3	5	5	5
Cornstarch		15	15	15
Commercial sugar		45.2 <sup>1</sup>	43.2 <sup>2</sup>	41.8 <sup>2</sup>
Protein		17.8 <sup>1</sup>		
Protein hydrolysates			20.8 <sup>2</sup>	
Protein hydrolysates after HgCl <sub>2</sub> pptn.				22.2 <sup>2</sup>
Cystine (E. & A., C.P.)			00.1	00.1

<sup>1</sup> This was not the same for the control and experimental diet because the proteins used did not contain the same percentage of nitrogen.

<sup>2</sup> The different diets varied as the nitrogen content of the hydrolysates. The figure given represents an average figure, but not the percentage in every diet. The material was being prepared while the feeding was in progress so the weight of the hydrolysates and sugar varied somewhat depending upon the percentage of nitrogen in the hydrolysates.

Fourteen and five-tenths (14.5) per cent of the protein ( $N \times 6.25 \times 100$ ) in the toxic wheat diet is in the whole wheat added. All other diets, except the control whole wheat diet, contained the same amount of protein nitrogen as this diet. This difference in the nitrogen in the protein and hydrolysates necessitated varying another constituent of the diet. The sugar was the constituent varied.

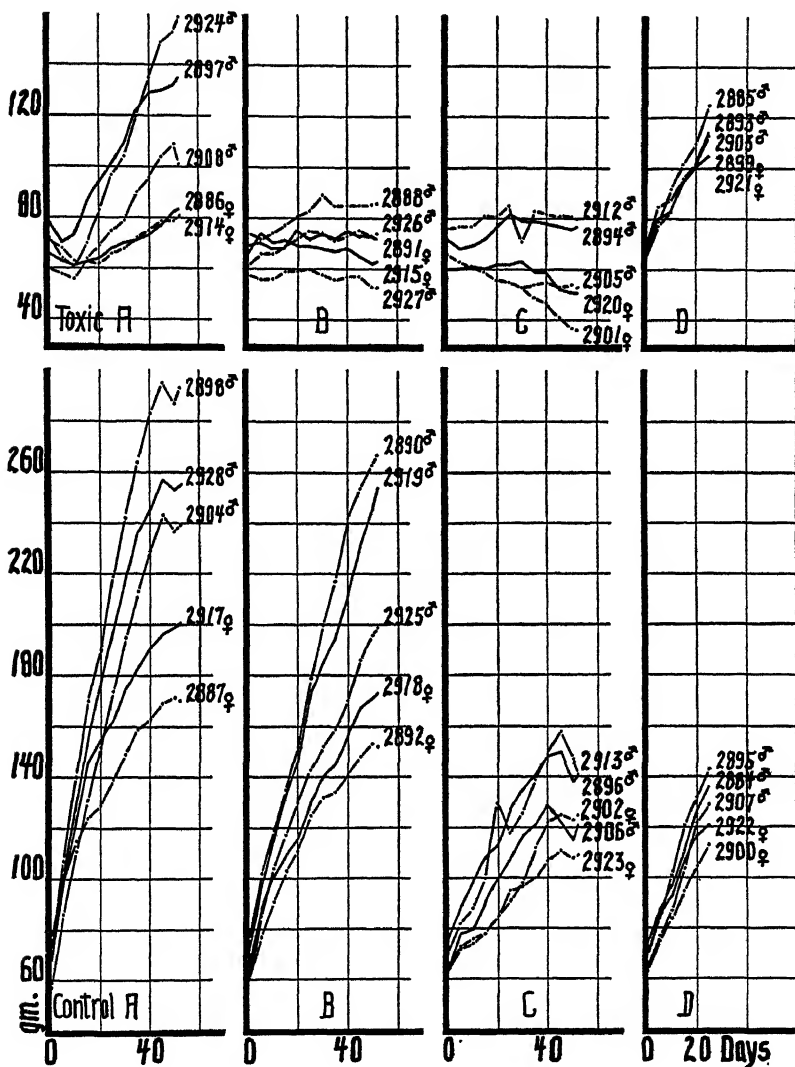
The average nitrogen content for the hydrolysates was approximately 11.2 per cent and for the amino acids resulting from mercuric chloride precipitation 10.4 per cent. It is realized that this is somewhat low; however, the weight of a protein hydrolysate increases over that of the protein, some of the ammonia nitrogen may have been lost and the hydrolysates were contaminated with small amounts of salt. The nitrogen content of the toxic protein was 13.0 per cent and of the control protein was 13.8 per cent. These figures seem to be very low, indicating that a pure protein was not used. Mitchell and Hamilton ('29) give 17.66 and 17.49 per cent,

respectively, for gliadin and glutenin, the chief proteins in wheat gluten. The proteins were purified by the method of Sandstedt, and Blish ('33). Both were taken from wheats of high protein content, 17.66 per cent for the toxic (582) wheat, and 17.5 per cent for the 'normal' (648) wheat. The factor 6.25 was used to convert nitrogen to protein. It appears that the method of Sandstedt and Blish did not separate all the protein from the starch. Further purification by dispersing the protein in alkali did not seem advisable because it has already been shown that the selenium in toxic proteins is unstable in alkaline solution.

Albino rats of Wistar Institute origin were the experimental animals used. They had been weaned on the twenty-first day and maintained on McCollum's diet I (Burr and Burr, '29) for 7 days. They were then divided into eight groups so that litter mates and the same number of males and females were in each group, and placed in individual cages described by Franke and Franke ('34). The average weight of the eight groups did not vary 0.5 gm. from the mean. The rats were weighed every 5 days and the food consumption recorded every 2 days.

Individual growth curves for the eight groups of series 101 are shown on figure 1.

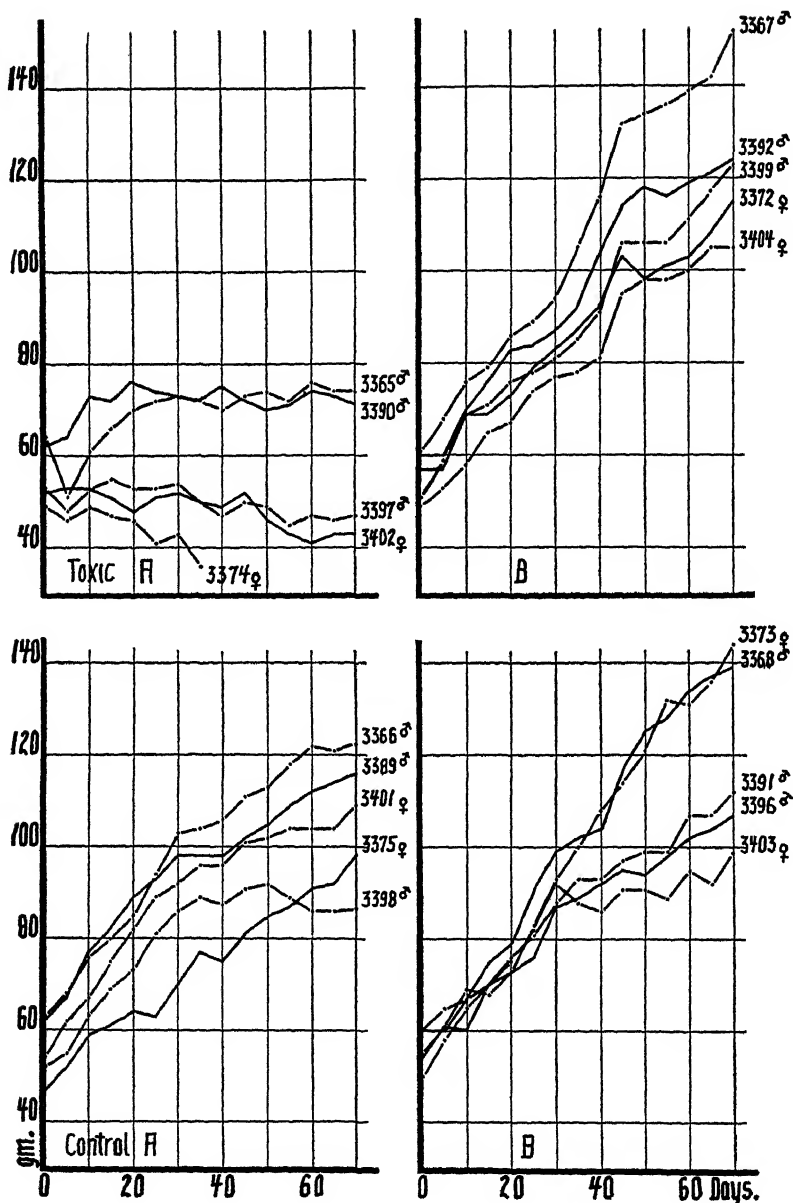
Due to the results obtained in the toxic and control groups C and D it seemed advisable to duplicate these four groups. The toxic hydrolysate which had the selenium removed appeared to be non-toxic; however, it was felt that a period of 25 days was not long enough to warrant conclusions. Furthermore, better growth was obtained in control group D, which had part of the amino acids removed by mercuric chloride precipitation, than in control group C. Also accurate food consumptions were not obtained during the first 20 days of series 101 on the groups receiving amino acid diets. This difficulty was overcome by adding water to the diet and making it into a thick soup when it was fed. Rose (personal communication) obviates this difficulty when feeding amino acid diets by using diets with a high fat content.



P-1 S-101

Fig. 1 Individual growth curves for series 101.

(Toxic) groups		(Control) groups	
A	Whole wheat	A	
B	Protein	B	
C	Hydrolysate	C	
D	Hydrolysate after HgCl <sub>2</sub> precipitation	D	



P-2 5-112

Fig. 2 Individual growth curves for series 112.

(Toxic) groups

A

Hydrolysate

B

Hydrolysate after HgCl<sub>2</sub>  
precipitation

(Control) groups

A

B

Series 112 was therefore planned. Four groups of rats were fed diets similar to those given groups C and D in series 101. These groups are designated as toxic groups A and B and control groups A and B (series 112). The following alterations of the method used in preparing the amino acids were made, otherwise the same experimental procedure was followed. The hydrolysates were neutralized with solid sodium carbonate instead of sodium hydroxide, and silver sulfate prepared in this laboratory was used to remove the chlorine from the filtrate after the mercuric chloride precipitation. The sulfate was then removed with barium hydroxide following the precautions already mentioned.

Individual growth curves for these groups which extended to the seventieth day are shown in figure 2.

All animals in both series were killed at the end of the experimental period and autopsies made. In some cases hemoglobin determinations were made following the method already described by Franke and Potter ('34).

#### DISCUSSION

The three control groups A, B and C of series 101 show clearly the nutritional values of synthetic and natural diets. The poorer growth rate using the protein and hydrolysate diet is clearly shown. The depression of growth after the fortieth day in the group fed the control hydrolysate diet (group C) may have been due to a slight amount of barium in the diet. It was found that sulfuric acid had to be present in excess to completely remove the barium. Rapport and Beard ('27) observed difficulty with barium toxicity when barium hydroxide was used to remove sulfuric acid from hydrolysates used in animal feeding.

There were no deaths in series 101 although this grain sample (laboratory no. 582) is one of the lethal samples reported by Franke ('34 a) in which over 70 per cent of the animals died before the sixtieth day of experimentation. This may have been due to the fact that the animals averaged 2 to 3 days older when placed on experiment than those reported by Franke. It has been shown (unpublished data)

that older animals have a greater resistance to the toxicity of selenium containing grains.

If depression of growth may be taken as a measure of toxicity it is quite evident that the protein is just as toxic after it has been separated from the wheat, dispersed in acetic acid and re-precipitated. The results here indicate that the protein diet is more toxic; however, it should be observed that the growth of the control group B is not as good as that in the control group A. Other feeding trials using toxic proteins indicate that the toxicity is not reduced by the method employed in its separation.

It is difficult to state whether or not the hydrolysate diet (group C) is as toxic as the whole wheat and protein diets. Growth was profoundly suppressed, but the control group also showed marked growth depression when compared with the groups receiving control wheat and control protein diets. The results suggest that the toxicity of the hydrolysate, due to the toxicant in the original grain, had been reduced.

The alkaloidal test indicated that the selenium contents of the toxic diets fed to groups A, B, C (series 101) and toxic group A (series 112) were the same. The method described by Robinson et al. ('34) gives 31 p.p.m. of selenium for the whole grain, 120 p.p.m. of selenium for the toxic protein, and 100 p.p.m. of selenium in the toxic hydrolysate. If these figures are correct, there is more selenium in the toxic grain diets than in the toxic protein or hydrolysate diets. The protein diets contain more selenium than the hydrolysate diets but the difference is hardly significant. It has been shown (Painter and Franke, '35 a) that considerable selenium is in the insoluble humin when toxic proteins are hydrolyzed with acids.

In both series better growth resulted in the control groups with the diet containing the hydrolysate after part of the amino acids had been removed by mercuric chloride precipitation than in the groups whose diet contained the untreated hydrolysate. The obvious question is: Did the mercuric chloride precipitation remove some growth depressant or toxicant present in protein hydrolysates? Or were the results

due to the removal of something rendering the diet more palatable? The change in color of the hydrolysate after the mercuric chloride precipitation indicated that much of the soluble humin had been removed. Undoubtedly the gustatory effect of protein hydrolysates in diets is a contributing factor in the failure of animals to respond favorably to these diets.

The necropsy of the animals revealed the usual symptoms produced by toxic foodstuffs. The severe pathological disturbances (Franke, '34 a) were not as acute in the animals receiving a toxic protein hydrolysate diet as in the animals receiving the toxic wheat and toxic protein diets. The external appearance of the animals in toxic group C (series 101) and toxic group A (series 112) was typical of that generally produced by toxic foodstuffs. The animals on toxic diets were very thin and in general appeared emaciated. All groups receiving control foodstuffs appeared normal except that those receiving the diets containing amino acids were much smaller. This was also true of the animals receiving toxic protein hydrolysates from which the selenium had been removed. They did not exhibit the typical pathological disturbances observed in the other groups.

Several hemoglobin determinations did not reveal any abnormally low levels in rats fed toxic protein hydrolysates which Franke and Potter ('35) observed in rats fed toxic grain. Only a few samples of blood were taken when the animals were killed and these were not from moribund animals. The two lowest levels obtained in series 112 were rats no. 3397 and no. 3402, which had hemoglobin levels of 14 and 12.6 gm. per 100 cc. blood, respectively. Those animals in the control group and protein hydrolysate with selenium removed had normal levels.

The voluntary restriction of food consumption observed (Franke, '34 a) in animals fed toxic grain diets was observed in the animals fed toxic protein hydrolysate diets. In series 112 there is a direct correlation between growth and food consumption. The available records of series 101 indicate that the growth rates and food consumption were in direct proportion. The average food consumption in grams per day



for the toxic groups A and B (series 112) was 3.32 and 6.33 and for the control groups A and B was 5.49 and 6.39. It is evident that the restriction of food consumption is due to the toxicant present in the whole wheat, protein and hydrolysate diets.

Both series 101 and 112 prove without question that the removal of selenium by the method described removes the toxicant present in toxic protein hydrolysates. This is clearly shown by comparing toxic groups C and D of series 101 and toxic groups A and B of series 112. The growth rate, external appearance of the animals, symptoms observed at autopsy and response to the diets all indicate that the toxicant was removed by the mercuric chloride precipitation so completely that the amount left was innocuous. Either selenium in some compound (or compounds) is the sole toxicant or other toxicants, if present, are likewise precipitated by mercuric chloride. The results obtained do not prove that selenium is the sole toxicant present in toxic foodstuffs but since no toxicant was removed from either toxic proteins or their hydrolysates by organic solvents, nearly all known plant toxicants are eliminated. Observations feeding selenium salts (Franke and Potter, '35) indicated that their action on the animal body is very similar to that of toxic grains, although the symptoms produced were not exactly or consistently the same.

#### SUMMARY AND CONCLUSIONS

The effect of chemical treatment of the proteins from toxic wheat has been studied by animal feeding. Growth curves of animals fed diets containing whole grain, protein, protein hydrolysate and protein hydrolysate after mercuric chloride precipitation are shown.

From the results obtained by the separation trials and feeding series, the following conclusions are made:

1. The sulfuric acid hydrolysates of toxic proteins are toxic.
2. Mercuric chloride precipitation of toxic protein hydrolysates (under the proper conditions) precipitates the selenium compound (or compounds) so nearly completely that the filtrate is innocuous when fed to albino rats.

3. Mercuric chloride precipitation removes something from sulfuric acid hydrolysates which directly or indirectly inhibits growth.

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# THE RELATION BETWEEN THE VITAMIN A AND D INTAKE BY THE HEN AND THE OUTPUT IN EGGS<sup>1</sup>

WALTER C. RUSSELL AND M. W. TAYLOR

*Department of Agricultural Biochemistry, New Jersey Agricultural Experiment  
Station, and Rutgers University, New Brunswick*

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Early in the history of vitamin studies McCollum and Davis ('13) and Osborne and Mendel ('14) showed that egg yolk contained a growth-promoting substance which later came to be known as vitamin A. Probably the earliest demonstration of the influence of the vitamin A content of the ration on the amount of this factor in the egg was by Bethke, Kennard and Sassaman ('27). Mellanby ('21) and later Hess ('23) reported that egg yolk possessed antirachitic properties. That the antirachitic potency of egg yolk could be increased by exposure of the hen to ultraviolet light was shown almost simultaneously by Hart, Steenbock and associates ('25) and by Hughes, Titus and Moore ('25). Bethke and co-workers ('27) confirmed this finding and added the observation that the antirachitic potency of egg yolk could be influenced by the amount of the factor in the ration.

The principal objective of this investigation was the determination of any relationship between the vitamin A and D intake and the quantity of these factors which appears in the eggs.

<sup>1</sup>Journal series paper of the New Jersey Agricultural Experiment Station, Department of Agricultural Biochemistry. Presented before the Division of Biological Chemistry of the American Chemical Society in Cleveland, Ohio, September, 1934.

## EXPERIMENTAL

*Feeding plan.* Three groups of four hens each were housed in individual compartments of a fattening battery on December 18, 1933. They were just entering their first laying period and during the preceding summer had been on range until August 1st, after which time they were in open front houses. The cages were equipped with specially constructed feeding cups which permitted accurate measurement of feed consumption. Mash feed, table 1, was available at all times but the scratch grain was given daily in weighed amounts. Although the original plan was to control the supply of scratch grain so that equal amounts of mash and scratch would be consumed daily, on account of the lack of exercise it was considered advisable to limit the allowance of scratch grain to approximately one-half the ad libitum mash consumption. Grit and oyster shell were fed separately.

*Vitamin A and D assays.* The vitamin A content of the cod liver oil, yellow corn, and egg yolk was determined essentially by the Sherman and Munsell ('25) method. Since the completion of this work, there has come into use the U. S. P. X 1934 revision (Interim Revision Announcement, '34) of the method for the assay for vitamin A in cod liver oil. This method is the same in principle as the procedure described by Sherman and Munsell except that a 4- instead of an 8-week assay period is used and a reference oil whose potency has been agreed upon is employed as a standard. Since a number of laboratories are using this reference oil, results comparable with those of other workers can best be obtained by referring the rat growth response at 4 weeks to a dosage-response curve for the reference oil and expressing the potency in terms of U. S. P. X 134 units. Unless otherwise stated the U. S. P. X 1934 unit is used in this publication.

Carotene was determined colorimetrically by the method developed originally by Willstätter and Stoll ('13) and modified by Russell, Taylor and Chichester ('35).

The value in biological units assigned to alfalfa meal was based upon its carotene content, at least a rough correlation

having been shown to prevail between the carotene content and the vitamin A value determined biologically (Russell, Taylor and Chichester, '34). It is recognized that the vitamin A potency of alfalfa meal and yellow corn, particularly if the latter is ground, will decrease during storage, but the values assigned to corn and alfalfa meal are considered to be typical of those products.

The egg yolk was fed suspended in a 1 per cent sodium chloride solution as a separate supplement, 1 to 2 cc. of the suspension being the volume which contained 1 dose. It was found convenient to draw the egg yolk into a Luer syringe in which condition it could be preserved in the refrigerator with little chance of spoilage because the yolk at the tip of the syringe hardened to a self seal. Eggs collected from the groups of four hens each during February were not pooled but were broken as required for the preparation of supplements, the number of eggs used from each hen being proportional to the number produced. Likewise eggs were broken as required for the assay in the case of individual hens.

The vitamin D potency of the cod liver oil and egg yolks was determined by the line test method essentially as originally described by McCollum, Simmonds, Shipley and Park ('22), and more recently used by the Wisconsin Alumni Research Foundation, and expressed in Steenbock units. The substances under examination were admixed with the ration. After the completion of this work, the U. S. P. X 1934 revision of the method for the assay for vitamin D in cod liver oil came into use. This method is essentially the same as that of the Wisconsin Alumni Research Foundation but it involves the use of a reference oil whose potency is expressed in U. S. P. X 1934 units and which has been agreed upon as a standard by a number of laboratories. In order to convert Steenbock units to U. S. P. X 1934 units the factor 3.2 was used, 1 Steenbock unit being equivalent to 3.2 U. S. P. X 1934 units for this animal colony.

## RESULTS AND DISCUSSION

*Vitamin A studies*

The rations, table 1, were designed for the study of the effect of an increase in vitamin A concentration of the ration on egg production, potency of egg yolk, and percentage output of the factor in the eggs. The concentration of vitamin A in the ration could have been markedly increased by the addition

TABLE 1  
*Composition of rations*

	VARIABLE CONSTITUENTS OF THE MASH		
	Group 1	Group 3	Group 4
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Yellow corn	0.0	32.0	27.0
White corn	32.0	0.0	0.0
Alfalfa meal	0.0	0.0	5.0

*Constant constituents of the mash*

Wheat bran, 18.5 per cent; wheat middlings, 18.5 per cent; pulverized oats, 9.0 per cent; meat scrap, 18.0 per cent; oyster shell, ground, 3.0 per cent, and salt (NaCl), 1.0 per cent. One part of cod liver oil no. 2543 added per 100 parts of mash.

	SCRATCH GRAIN		
	Group 1	Group 3	Group 4
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Yellow corn, cracked	0	32	50
White corn, cracked	50	18	0
Whole wheat	50	50	50
One part of cod liver oil no. 2543 added per 100 parts of scratch.			

of carotene but it was desired to keep the vitamin A potency within the limits which are possible with the use of practical and commonly used feeds. The vitamin A potency of the constituents of the ration carrying this factor and of the egg yolks are displayed in table 2. The weight of an egg yolk, 16 gm., was adopted after the weighing of a number of the yolks used in the experiment. The percentage output of the factor in the eggs expresses the relation between intake and

output, but does not take into consideration the possibility that some of the vitamin A in the eggs may have come from the body stores.

The number of individuals under observation, table 3, was small and it is recognized that marked differences in performance of the individuals of a group of hens on the same

TABLE 2  
*Vitamin A content of rations and eggs*

CONSTITUENT		<u>VITAMIN A PER GRAM</u> U. S. P. X 1934 UNITS	
Individual constituents			
Cod liver oil no. 2543		1400	
Yellow corn		7	
Alfalfa meal		120	
Rations			
Group 1			
Mash		1400	
Scratch grain		1400	
Group 3			
Mash		1625	
Scratch grain		1625	
Group 4			
Mash		2200	
Scratch grain		1750	
Eggs			
FEBRUARY, 1934	<u>VITAMIN A PER 16 GM. YOLK</u> U. S. P. X 1934 UNITS	MAY, 1934	<u>VITAMIN A PER 16 GM. YOLK</u> U. S. P. X 1934 UNITS
Group 1	540	Group 1, hen no. 100	600
Group 3	500	Group 3, hen no. 54	800
Group 4	540	Group 4, hen no. 48	680

ration are always found. However the results permit the calculation of the amount of vitamin A in the eggs as a percentage of that consumed.

The number of units of the factor ingested by group 4 in February was almost twice that by group 1 and the number of eggs produced by group 4 was of the order of twice that



by the latter group. Since the vitamin A potency of the eggs was the same for these two groups, the percentage of the vitamin in the ration which appeared in the eggs was essentially the same for both groups, 16 and 17 per cent, re-

TABLE 3

*Vitamin A intake in the feed and output in the eggs. February, 1934.*  
U. S. P. X 1934 units

HEN NO.	FEED INTAKE		EGGS LAID	VITAMIN A INTAKE			VITAMIN A OUTPUT IN EGGS	VITAMIN A OF RATION WHICH APPEARED IN EGGS
	Mash	Scratch		Mash	Scratch	Total		
Group 1								
	<i>gm.</i>	<i>gm.</i>		<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>per cent</i> <sup>1</sup>
27	1225	650	5	17,150	9,100	26,250	2,500	10
61	1280	650	12	17,920	9,100	27,020	6,000	22
67	520	650	2	7,280	9,100	16,380	1,000	6
100	1165	500	10	16,300	7,000	23,300	5,000	21
Total	4190	2450	29	58,650	34,300	92,950	14,500	Average 16
Group 3								
10	890	650	0	15,460	10,560	25,020	0	0
17	1090	650	4	17,720	10,560	28,280	2,160	8
24	860	650	6	13,990	10,560	24,550	3,240	13
54	1765	650	8	28,700	10,560	39,260	4,320	11
Total	4605	2600	..	74,870	42,240	117,110		
Total for laying hens	3715	1950	18	60,410	31,680	92,090	9,720	Average 11
Group 4								
46	1610	650	17	33,320	11,380	44,700	9,180	21
49	820	650	6	18,050	11,380	29,430	3,240	11
74	1790	650	20	39,400	11,380	50,780	10,800	21
96	1185	650	8	26,100	11,380	37,480	4,320	11
Total	5305	2600	51	116,870	45,500	162,390	27,540	Average 17

<sup>1</sup> Percentages are given to nearest whole number.

spectively. The individual hens studied in May, table 4, consumed more ration and consequently more vitamin A than they did in February and produced more eggs. Also the vitamin A potency of the yolks of eggs from individual hens was higher in May than the values obtained for the pooled eggs in

February. The net result of the changes in vitamin A consumption, egg production and possible increase of egg yolk potency (in the case of individual birds), was an increase in the percentage of vitamin A of the ration which appeared in the eggs in May. The percentage values were 32 and 27 per cent, respectively, for groups 1 and 4 for May as compared with 16 and 17 per cent in February. Group 3 was not producing as well as nos. 1 and 4 in February and the percentage output of the factor in the eggs was low, 11 per cent, although the average consumption of vitamin A per hen was greater than that of group 1. In May the representative of group 3 showed a percentage output of the vitamin of the same order

TABLE 4

*Vitamin A intake in the feed and output in the eggs by individual hens. May 1934.  
U. S. P. X 1934 units*

GROUP NO.	HEN NO.	FEED INTAKE		EGGS LAID	VITAMIN A INTAKE			VITAMIN A OUTPUT IN EGGS	VITAMIN A OF RATION WHICH APPEARED IN EGGS
		Mash	Scratch		Mash	Scratch	Total		
		<i>gm.</i>	<i>gm.</i>		<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>per cent</i>
1	100	2745	620	25	38,500	3,700	47,200	15,000	32
3	54	2960	620	22	48,200	10,100	58,300	17,600	30
4	46	1675	620	19	36,900	10,850	47,750	12,920	27

as that of the other groups. These percentages are of the same order as a number of those reported by Sherwood and Fraps ('32), although in a few cases they reported higher values.

At the end of the experiment the vitamin A potencies of the livers of the eleven surviving birds were determined by the antimony trichloride method according to Moore ('30) and the values expressed as blue units (Moore) per gram (table 5). When the blue unit values are arranged according to the decreasing order of the number of eggs laid by individuals during the 5-month period, they are found to fall, with one exception, hen 67, pen 1, in the reverse order. Thus the best producers had the lowest A potency per gram of liver in a given pen. Presumably the highest producers were drawing

more heavily on their vitamin A stores than the lower producers and thus would end a laying season with a low vitamin A store in the body.

### *Vitamin D studies*

In an earlier experiment, 1932-1933 (unpublished data), hens were fed a ration similar to that employed in this experiment but they were kept in an open front house and 2 per

TABLE 5  
*Relation between the vitamin A in the liver and egg production*

HEN NO.	EGGS LAID IN 5 MONTHS	BLUE UNITS (MOORE) PER GRAM OF LIVER
Group 1		
100	84	24
61	61	Neg.
27	16	435
67	14	350
Group 3		
54	69	107
17	48	121
24	43	182
10	13	332
Group 4		
74	102	42
46	92	88
96	27	200

cent of the same cod liver oil employed in the present experiment was incorporated in the mash. In January, 1933, the eggs from these birds contained 16 U. S. P. X 1934 units of vitamin D per yolk, table 6; essentially the same potency that was found in February in the present experiment. The potency of egg yolk in May, 1933, was 32 units per egg whereas in June, 1934, the number of units per yolk was less than 13, probably of the order of 6. The difference between the potencies observed in May, 1933, and June, 1934, was undoubtedly due to the sunlight received by the hens in the open front

house as compared with the lack of sunlight when the hens were caged indoors in the present experiment. The data suggest that sunlight is more effective in increasing the anti-rachitic potency of egg yolk than the amount of cod liver oil ordinarily used in the case of the hen; a suggestion which is not in agreement with the conclusions of Branion, Drake and Tisdall ('35).

TABLE 6

*Vitamin D intake in the feed and output in the eggs. U. S. P. X 1934 units*

				VITAMIN D PER 16 GM. YOLK	
January, 1933				16	
May, 1933				32	
February, 1934				13	
June, 1934				Less than 13	

Group 4, February, 1934								
HEN NO.	FEED INTAKE		EGGS LAID	VITAMIN D INTAKE			VITAMIN D OUTPUT IN EGGS	
	Mash	Scratch		Mash	Scratch	Total		
	<i>gm.</i>	<i>gm.</i>		<i>units</i>	<i>units</i>	<i>units</i>	<i>units</i>	<i>per cent</i> <sup>1</sup>
46	1510	650	17	1220	530	1750	218	12
49	820	650	6	660	530	1190	77	6
74	1790	650	20	1440	530	1970	256	13
96	1185	650	8	950	530	1480	103	7
Total	5305	2600	51	4270	2120	6390	654	Average 10

Vitamin D in the ration—the mash and scratch grain each contained 80 units of vitamin D per 100 gm.

<sup>1</sup> Percentages are given to nearest whole number.

As shown in table 6, 10 per cent of the vitamin D consumed appeared in the eggs in the case of group 4. The values for the antirachitic potency of egg yolk (on a comparable unit basis) are somewhat lower than those reported by Branion and associates ('35).

Experiments in this and other laboratories have shown that less than 4 per cent of the vitamin A (Baumann, Steenbock, Beeson and Rupel, '34, and Russell, Taylor, Chichester and Wilson, '35) and less than 2 per cent of the vitamin D (Russell,

Wilcox, Waddell and Wilson, '34 and Krauss, Bethke and Wilder, '34) of the cow's ration appear in the milk when adequate amounts of these factors are fed. In the case of the hen, 11 to 32 per cent of the vitamin A and 10 per cent of the vitamin D of the ration were found in the eggs. Hence the latter species is more efficient as a transmitter of these two vitamins. The fact that the fat content of the edible portion of eggs is 10 per cent as compared with 3.5 to 4.0 per cent for milk may be the explanation of the difference, because both of these vitamins are fat soluble and might be expected to be present in larger amount in the medium containing the higher percentage of fat.

We are indebted to Dr. D. F. Chichester of this laboratory for the determination of the blue units of vitamin A in the livers of the birds.

#### SUMMARY

The output of vitamin A in eggs, calculated as the percentage of that consumed, varied from 11 to 32 per cent, and was determined by the number of units of the factor consumed, the number of eggs produced and the potency of the yolk. The highest percentages were obtained during high production.

The liver vitamin A of the hens varied inversely with egg production.

In the case of vitamin D the amount of the factor which appeared in the eggs was 10 per cent of that consumed.

The data suggest that sunlight is more effective in increasing the antirachitic potency of egg yolk than the amount of cod liver oil ordinarily fed.

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# A STUDY OF THE NUTRITIVE VALUE OF MUSHROOMS<sup>1</sup>

F. W. QUACKENBUSH, W. H. PETERSON AND H. STEENBOCK

*Department of Agricultural Chemistry, University of Wisconsin, Madison*

FIVE FIGURES

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## INTRODUCTION

The nutritional value of the mushroom has long been a subject of speculation. Although most of the interest has been directed to the protein fraction, little can as yet be said with certainty regarding the actual proteins present and their digestive and nutritive properties. Less extensive studies by Orton, McCollum and Simmonds ('22), Hara ('23), Steidle ('24), and Scheunert and Reschke ('31) on the vitamins in mushrooms have revealed the presence of vitamin A activity in certain varieties, conflicting evidence on the vitamin B content, and little or no vitamin C. Sumi ('33) has found a considerable amount of ergosterol as the precursor of a vitamin D, and Scheunert and Reschke ('31) a vitamin D itself. In a comparison of the nutritional value of mushrooms with that of vegetables, Sabalitschka ('31) has reviewed the occurrence of various other dietary constituents of mushrooms, such as fat, sugar, nitrogen-free extract, fiber and ash.

The high nitrogen content (König, '03) of the mushroom, usually found to be above 5 per cent and sometimes as high as 10 per cent, led early writers to regard it as an excellent source of protein. However, Böhmer (1882) found that only

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64 per cent of the nitrogen of *Agaricus campestris* was precipitable with  $\text{Cu}(\text{OH})_2$ . He therefore considered the remaining 36 per cent to be non-protein in character. Mörner (1886) reached a similar conclusion using solubility in 85 per cent alcohol as a criterion for non-protein-nitrogen. Of the total nitrogen in samples of *Agaricus campestris* (cap 7.38 per cent and stem 6.02 per cent nitrogen), he found only 66 per cent insoluble in 85 per cent alcohol. Of this protein-nitrogen, 25 per cent was not digested in vitro by successive treatments with gastric and pancreatic extracts, indicating an 18 to 20 per cent content of digestible protein, calculated on the dry basis. Mendel (1898), using Mörner's technic with mushrooms of lower nitrogen content, found the alcohol insoluble digestible protein of several varieties to be less than 10 per cent of the total dry tissue. In attempting to characterize the non-protein nitrogen he pointed out that Winterstein (1894) was unable to make a nitrogen-free 'cellulose' preparation from *Boletus edulis*. Winterstein's purified 'cellulose' preparation contained 5.5 per cent nitrogen, and upon hydrolysis yielded mainly glucosamine. Mendel suggested that a considerable part of the nitrogen in mushrooms probably exists in this form.

The measurement of the digestibility of mushroom nitrogen by actual feeding trials was attempted by Saltet (1885). He found that a normal human was able to digest 69 per cent of the nitrogen in a sample of *Agaricus campestris*. Recently Skinner, Peterson and Steenbock ('33) found that albino rats digested 71 per cent of the nitrogen present in a sample of the same variety.

The available data concerning the vitamin B content of mushrooms are confusing, since they fail to differentiate clearly the various B components. Orton, McCollum and Simmonds ('22) fed a sample of dried *Agaricus campestris* at a 9 per cent level to two rats which had ceased to grow on a diet lacking in the vitamin B complex. The growth response was immediate, with an average weekly gain of 10.8 gm. per rat over a 5-week period. Hara ('23) found *Boletus edulis*

fully as effective as a high grade of yeast in restoring growth in rats on a ration deficient in 'vitamin B.' Weekly growth rates of 13 to 16 gm. were obtained with daily supplements of 0.3 to 0.5 gm. of dried mushrooms over a 3-week period. A sample of *Agaricus campestris* was only slightly inferior. Scheunert and Reschke ('31) in their recent survey of the vitamins in mushrooms have reported assays of several varieties, both in raw and cooked condition. They concluded that mushrooms are deficient in vitamin B complex, particularly in the vitamin B<sub>2</sub>(G) component. Three to 6 gm. of either raw or cooked material were found necessary to produce an appreciable growth response in depleted rats. Unfortunately they failed to state the ingredients of their basal diets.

The foregoing review clearly illustrates the desirability of further investigating the nutritionally important components of the mushroom. It was with the hope of clarifying our knowledge of the nutritional properties of mushroom protein, in particular, that the present research was undertaken. Complications in the direct attack, which made their appearance early in the work, led to a detailed investigation of the vitamin B complex.

#### EXPERIMENTAL

*General technic.* The common mushroom, *Agaricus campestris*, has been used throughout these experiments. Commercial 'buttons' about 1 to 2 inches in diameter were purchased, usually in 100-pound lots. In early experiments the mushrooms were chopped, frozen immediately, and stored in this condition until needed. Each week a portion of the frozen material was finely ground, placed in a thin layer before a fan to remove a part of the water, and then incorporated into the diets. Under this treatment the tissue turned dark brown in color. It was soon found that a much better appearing product resulted if the fresh mushrooms were sliced and spread in thin layers before a fan to dry at room temperature. The dried material could then be finely ground and stored at

room temperature without difficulty. However, the samples prepared in these two ways revealed no apparent difference in nutritive properties. The casein used was a commercial acid casein which had been purified by repeated washing for a week with water slightly acidified with acetic acid.

Some 200 male rats have been utilized in these experiments. Except when otherwise stated they were placed on experiment at weaning age (3 to 4 weeks) at a weight of from 50 to 60 gm. They were housed in individual cages with raised  $\frac{1}{2}$  inch mesh screen bottoms. Daily food consumption records were maintained for all animals.

In reporting the data we have adopted a uniform method as follows. All weights of mushrooms as well as other materials are given calculated to a dry basis. All supplements to basal diets have been made by replacing an equal weight of starch. Diets bear the same numbers as the groups to which they were originally fed. The units in all figures (group 23 is the only exception) are so chosen that the ratio of abscissa to ordinate is constant, regardless of scale. The numbers in parentheses accompanying each curve give the number of animals used to arrive at the average values of the graph.

*Protein studies.* Four groups of rats were fed diets containing 50 per cent of mushrooms, with and without added protein. Samples of the mushrooms themselves averaged 6.18 per cent nitrogen. The composition of the diets is given in table 1. All animals in groups 1 and 2 were fed ad libitum. The diets of groups 3 and 4 were constructed so as to contain respectively 5 per cent of wheat and corn proteins, in addition to the protein contained in the mushrooms. The animals on these two diets were kept on an equalized food intake determined by those rats, within their groups, which had the lowest intake.

The results of these experiments are presented in figure 1. It will be seen that when mushrooms served as the sole source of protein, as well as the sole source of vitamin B, the animals failed to grow. All died within 37 days. The addition

of 5 per cent of wheat or corn protein, or even 18 per cent casein, gave only slightly better results. The apparent improvement resulting with diet 2 as rats increased in age led us to start six more animals weighing in excess of 70 gm.,

TABLE 1  
*Composition of diets*

CONSTITUENTS	DIET NUMBER							
	1	2	3	4	5	6	7	12
Mushrooms (dry basis)	50	50	50	50				10
Cornstarch (autoclaved)	44	26			76	68	48	66
Salts 40	4	4	4	4	4	4	4	4
Cod liver oil	2	2	2	2	2	2	2	2
Casein (purified)		18			18	18	18	18
Yellow corn			40					
Corn gluten			4					
Wheat				44				
Yeast (autoclaved 5 hours)						8		
Butterfat							8	
Wheat extract dried on starch							20	

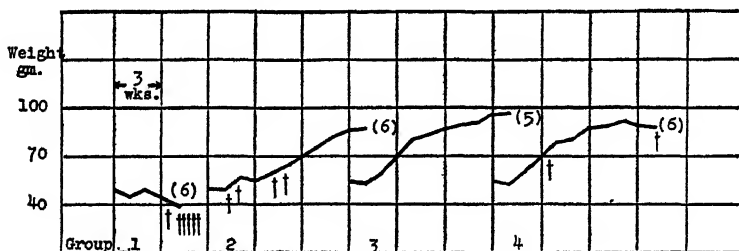


Fig. 1 Fifty per cent mushrooms as a source of protein for growth. Groups 2, 3 and 4 received in addition casein, corn, and wheat proteins, respectively. Parenthetical numbers give the number of individuals represented by each curve. Daggers indicate deaths.

using the same diet. The results were no better than those obtained with the 50-gm. rats.

The failure to obtain satisfactory growth even with a diet containing adequate protein exclusive of that contained in the mushrooms, together with the observation that food intake was low (5 to 8 gm.), led us to suspect that the mushrooms

might be deficient in vitamin B<sub>1</sub> or some other member of the B complex.

*Studies on the vitamin B content of mushrooms.* Therapeutic methods were employed in testing the mushrooms for the vitamin B complex, and B and G individually. The diet used in assaying for B has been described by Kemmerer and Steenbock ('33). The G assay was made with the technic of Bourquin and Sherman ('31). Table 1 gives the detailed composition of the basal diets; figure 2 the average growth curves of the animals.

The first assay was made for the B complex. When growth on the basal diet had definitely failed, the animals in group 5 were given mushroom supplements to the extent of 10 per cent and 25 per cent, respectively, of their diet. The graphs show that only a temporary growth response at a slow rate was obtained on either level. This failure led us to assay for B and G.

It will be seen from figure 2 that the B-depleted animals (group 6) responded somewhat to both 2 per cent and 5 per cent mushrooms. These animals were continued several weeks beyond the test period shown. Between 10 and 14 weeks after supplementation was begun all animals on the 2 per cent level had ceased to grow and died from polyneuritis; during the same period no polyneuritic symptoms were observed in any of the animals on the 5 per cent level. A 10 per cent level of mushrooms produced an average gain of 21 gm. per week. Since no better response was made on a 25 per cent level it was concluded that a diet containing 10 per cent mushrooms supplied an ample amount of vitamin B for growth.

Although the Bourquin-Sherman diet has the shortcoming that it does not produce consistently the skin changes which are considered by many to be due to a vitamin G deficiency, nevertheless this diet was believed to be the most satisfactory one available. Animals confined to this diet ceased to grow within 4 to 6 weeks but seldom exhibited clear cut symptoms of dermatitis such as sores about the mouth and paws. Supplementation of the diet with 2 per cent of mushrooms did not

improve growth consistently; neither did it intensify the skin symptoms. However, the addition of 5 per cent or more produced satisfactory growth; about 20 gm. per week, and quickly restored the animals to normality.

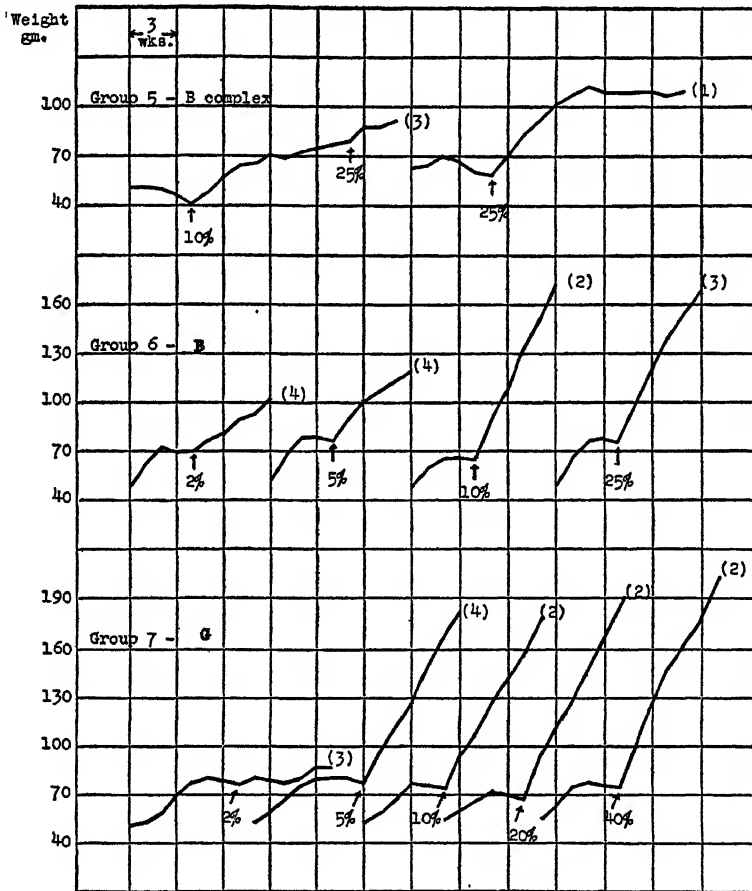


Fig. 2 Mushrooms as a source of the vitamin B complex, and of vitamin B and G. Arrows and percentages indicate mushroom supplements. Parenthetical numbers give the number of individuals represented by each curve.

It was concluded from these data that a diet containing 10 per cent of mushrooms supplies adequate amounts of both vitamins B and G. The results obtained with group 5 indicate,

however, that this level, when animals were dependent upon it as the sole source of the B complex, was by no means sufficient to produce normal growth. It appeared, therefore, that some factor, other than B or G, which was present in autoclaved yeast as well as in an alcoholic (80 per cent by weight) extract of whole wheat, must be added to either a 10 per cent or a 25 per cent level of mushrooms in order to supply adequately the whole vitamin B complex. The third phase of the work was designed to ascertain whether or not such a factor exists, and if so to study its occurrence and properties.

*Supplementary effect of various materials.* A group of animals (group 12) was started on the basal diet used in group 5, to which 10 per cent of mushrooms was added at the start. The animals ate sparingly at first but gradually increased their subnormal food intake during the first 6 or 8 weeks. Although a part of the group continued to grow slowly (4 to 10 gm. per week) many of the animals definitely ceased growing. These were given various dietary supplements of crude materials and extracts of crude materials.

The method employed in making the various alcoholic extracts was, with minor variations, that described by Bourquin and Sherman ('31) in the preparation of a wheat extract for their low vitamin G diet. It consisted of two successive extractions with several-fold volumes of alcohol (80 per cent by weight) followed by concentration of the extract in a vacuum at a low temperature. The concentrate was then poured on a suitable quantity of cooked starch and dried before a fan at room temperature. This 'activated starch' was incorporated directly into the ration.

Some results of these experiments are shown in figure 3. It will be seen that the dried liver and alfalfa extract proved to be very potent supplements. The wheat extract, autoclaved yeast, and brain tissue were slightly less effective. Although rat 79 failed to respond to the liver residue, a definite response was obtained when 2 per cent of the adsorbate from rice polishings replaced the 10 per cent liver residue. This led us to try a sample of crystalline B which had been

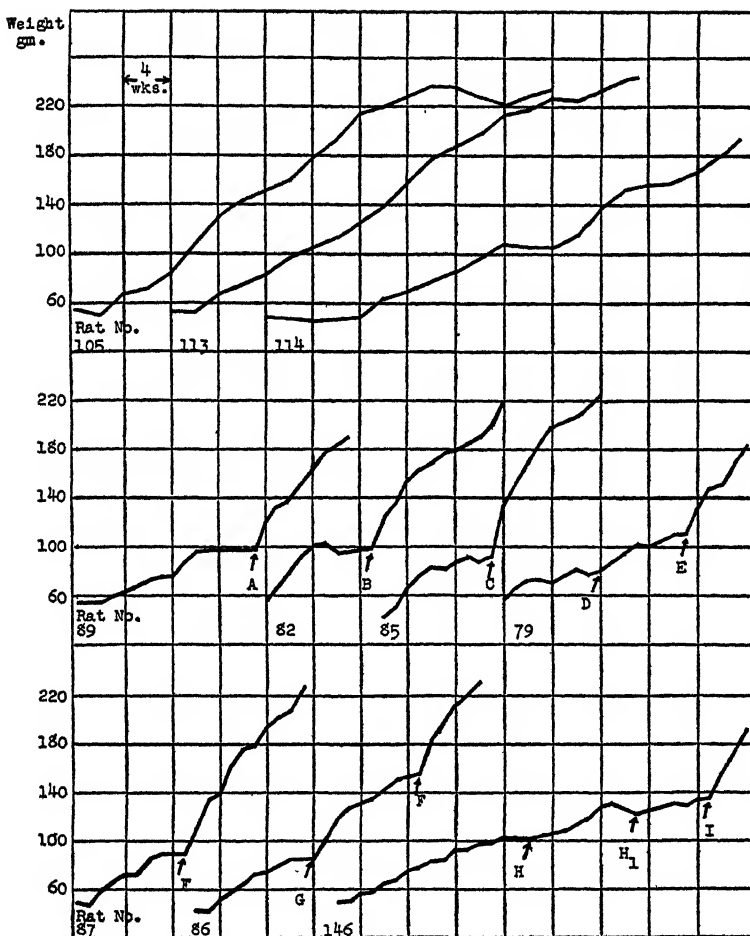


Fig. 3 The efficacy of various materials in supplementing diet 12, which contained 10 per cent of mushrooms as the sole source of vitamin B complex. The letters and arrows designate the supplements which are correspondingly listed below.

A, alcoholic (80 per cent by weight) extract of whole wheat in amount equivalent to 50 per cent of wheat in the diet.

B, 10 per cent Anheuser-Busch yeast (autoclaved 5 hours at 15 pounds' pressure).

C, 10 per cent vacuum dried fresh hog's liver.

D, 10 per cent of the autoclaved (10 hours) residue of fresh hog's liver from which the juice had been pressed.

E, 2 per cent of Fuller's earth adsorbate from rice polishing (Williams' B concentrate).

F, alcoholic extract of dried green alfalfa in amount equal to 20 per cent dry alfalfa in the diet.

G, 10 per cent dried fresh hog's brain (dried at 45°C.).

H, 2.5 gamma of crystalline B (Ohdake) daily (discontinued at H<sub>1</sub>).

I, 5 per cent vacuum dried fresh hog's liver.



obtained from Ohdake, 2.5 micrograms of which is equivalent to at least 20 mg. of the International Standard B preparation in vitamin B potency (Ohdake, '34). It had previously been found that 2.5 micrograms of these crystals gave a good growth response in an assay conducted with diet 6. The curve for rat 146 shows the negative result obtained in this test. Subsequent addition of 5 per cent of dried liver to the diet of the same animal produced a very definite response. Space does not permit the inclusion of graphs of all the data, but the results were as follows. Autoclaved wheat extract was as effective as the raw substance. Alcoholic extract of autoclaved yeast was inactive, and a similar extract of liver was somewhat less effective than the whole liver. Lower levels of the various materials gave poorer responses. Both 5 per cent alfalfa extract and 5 per cent whole dried alfalfa failed to give appreciable responses.

The curves for rats 105, 113 and 114 are typical of animals which continued to grow slowly on diet 12 even though no dietary supplements were given. In no case was coprophagy observed in animals receiving this diet. During the long period of the experiment these animals failed to develop gross pathological symptoms other than a general sluggishness and inactivity. Some of those which failed to grow developed priapism. At 39 to 40 weeks animals 105, 113 and 114 were autopsied. The internal organs appeared entirely normal.

The failure of these and numerous other animals receiving the same diet to cease growing or to show a definite pathology was somewhat puzzling. Strong evidence against the existence of a toxic factor was shown by the apparently normal condition of animals after long periods of mushroom feeding, as well as by their continued well-being on high intakes of mushrooms following supplementation with active materials. The low food consumption offered some evidence that the mushrooms lacked palatability. However, low food intake is also known to result with diets which are deficient in known nutritive essentials. The readiness with which the B- or G-depleted animals consumed large quantities of ration containing 25 per cent or 40 per cent of mushrooms, together

with the high supplementary value of such materials as alfalfa extract or fuller's earth adsorbate as against that of pressed liver residue, are further evidences opposing the view that mushrooms made the diets unpalatable. Attempts to improve the palatability and consumption of diet 12 by the addition of 10 per cent of butterfat were without success. Substitution of sucrose for cornstarch in the basal diet led only to greater variability of weekly gains.

*Fractionation of the mushrooms by extraction.* Booher ('33) found that a substance which would restore growth and cure dermatitis in rats suffering from G-deficiency was soluble in 94 per cent alcohol. Since, likewise, B can be extracted readily from wheat with 80 per cent alcohol, it was surmised that an extraction of mushrooms with alcohol might remove vitamins B and G and leave behind a considerable part of the factor which we were attempting to study. Accordingly, an alcoholic extract of dried mushrooms was made in the same manner as has been described for other materials. However, subsequent assays revealed that B and G remained almost quantitatively in the extracted mushrooms. A level of the extract equivalent to 25 per cent of mushrooms in the diet was found insufficient to give a growth response with either B- or G-depleted animals.

A water extract of the mushrooms was then prepared as follows: 1 kilo of dried and finely ground mushrooms was suspended in 5 liters of water and the mixture brought to boiling. After standing for half an hour the viscous material was filtered with a Büchner funnel, and the residue suspended in 3 liters of water and filtered again. The combined filtrate was reduced to a small volume in vacuo and dried on 1.5 kilos of dried cooked starch before a fan at room temperature. The dried material contained about 40 per cent of the original weight of the dried mushrooms. It was fed as a supplement to basal diet 5 at levels equivalent to 10 per cent, 20 per cent, and 40 per cent of mushrooms. The results are presented in table 2. They will be discussed in connection with other phases of the work which are presented in the same table.

*Further studies on supplementary substances; relationships between growth rate and amount of food consumed.* A series of trials was made in which various levels of dried yeast and mushrooms were fed, both singly and in combination with each other. Diet 5 was used as the basal diet. The results are summarized in table 2. During the 10-week period group 8, which received 8 per cent of dried yeast, reached an average weight of 299 gm., representing a weekly gain of 24.7 gm. per rat. Both food consumption and growth rate were markedly less when 10 per cent of mushrooms was fed together with 8 per cent of yeast (group 9). Group 12A, which received 10 per cent of mushrooms alone (diet 12), performed still less satisfactorily. Although an apparent slight advantage was obtained with 20 per cent of mushrooms, 40 per cent was inferior to either level, with no improvement resulting from the addition of 8 per cent of yeast. The data presented for groups 15, 16 and 17 show, however, that a reverse situation obtained with the water-soluble fraction of mushrooms. Animals receiving this material at a 10 per cent equivalent level definitely failed. Increasing levels gave increasing growth rates, the 40 per cent level being superior to any of the other diets containing only mushrooms as a vitamin B source.

The effectiveness of various B preparations in supplementing diet 12 was further investigated. A comparison of the data for groups 12a and 18 reveals that here, again, as with rat 146 above, no advantage was shown by rats receiving 2.5 micrograms of Ohdake's crystalline vitamin B. An acid-alcohol extract of yeast (Itter, Orent and McCollum, '35) fed at a level equivalent to 4 per cent of yeast in the diet, was only slightly better. An apparent improvement was obtained with groups 19 and 20 which received the International Standard B preparation and a Burroughs-Wellcome B concentrate from rice polishings, respectively, in amounts equivalent to 2 International units of vitamin B. The slightly better results obtained with the crude B carriers appeared not to be highly significant. Unfortunately the limited supply of materials prevented us from feeding them at higher levels. At the

termination of these trials one rat in group 20 was given diet 12 alone. During the next 4 weeks, its body weight fell 11 gm. The rice-polishings concentrate was then fed in a separate container at a level which was tenfold that previously fed. During the following 3 weeks the animal doubled its food intake and gained 79 gm. (26 gm. per week).

For comparison with other data presented, the mean growth rate of fifty-seven animals which had been confined to diet 12 is included in the table.

A comparison was made between diet 12 and several satisfactory growth-promoting rations in equalized feeding trials. The food intake of animals in group 11 was controlled by that of

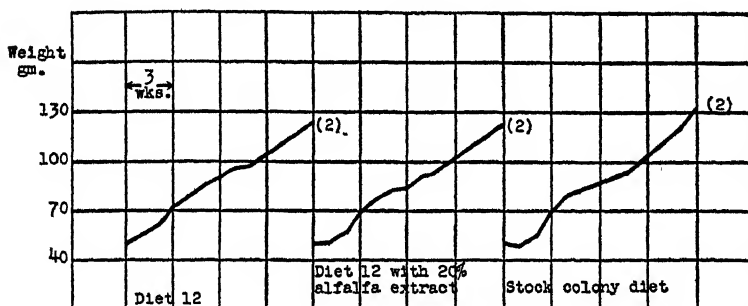


Fig. 4 A comparison of growth in equalized feeding experiments. Food consumption was controlled by the animals receiving diet 12.

group 12a; likewise in group 22 the food intake of animals receiving diet 12 determined the intake of those receiving other diets. It will be seen from the results shown in table 2 and group 22 in figure 4 that no marked differences in growth occurred, indicating that quantity rather than quality of the diet was the deciding influence upon growth. These data are only further verification of similar evidence shown in table 2, that those animals which ate the most food showed the best gains.

The curves for group 23 shown in figure 5 throw some additional light upon this subject. Animals weighing about 40 gm. each were placed in individual cages and received the stock colony diet (Steenbock, '23) for 6 days. By this time

all had become completely accustomed to their new environment and were eating 10 gm. of food daily. The diet was then changed. Two animals received diet 8 (8 per cent yeast) and

TABLE 2

*Comparison of growth on mushroom, yeast and supplemented mushroom diets.  
Experimental period 10 weeks*

GROUP NO.	SUPPLEMENT TO BASAL DIET 5	NUMBER OF RATS	FOOD CONSUMPTION (AVERAGE GRAMS PER RAT PER DAY)	MEAN BODY WEIGHT		
				Weekly gain per rat	Maximum	Final
8	8 per cent yeast	3	13.5	gm. 24.7	gm. 299	gm. 299
9	8 per cent yeast; 10 per cent mushrooms	2	8.1	13.5	185	185
10	8 per cent yeast; 40 per cent mushrooms	3 <sup>1</sup>	4.8	5.3	104	104
11	8 per cent yeast (food intake same as group 12a)	3	5.5	7.8	136	135
12a	10 per cent mushrooms	3	5.5	6.3	119	116
13	20 per cent mushrooms	3	5.4	8.3	136	136
14	40 per cent mushrooms	3	4.9	4.9	102	102
15	Water extract from 10 per cent mushrooms	3	4.8	3.8	99	91
16	Water extract from 20 per cent mushrooms	3	6.5	9.4	148	148
17	Water extract from 40 per cent mushrooms	3	6.7	10.9	161	161
18 <sup>2</sup>	10 per cent mushrooms; 2 units B <sub>1</sub> crystals (Ohdake)	2	5.8	7.0	116	111
19	10 per cent mushrooms; 2 units Int. Std. B <sub>1</sub>	3	6.8	11.0	160	160
20	10 per cent mushrooms; 2 units Burroughs-Wellcome B <sub>1</sub> concentrate	3	7.2	11.5	165	165
21	10 per cent mushrooms; 4 per cent yeast extract (Itter)	2	6.6	8.9	140	140
12 <sup>3</sup>	10 per cent mushrooms	57		5.0		95

<sup>1</sup> One animal died at 5 weeks.

<sup>2</sup> Nine-week period.

the other two received diet 12 for the following week. Average food consumption and body weight of each pair are shown in the figure. The animals receiving yeast continued to consume over 10 gm. of food and grew normally. Those receiving

mushrooms consumed successively an average of 8, 5 and 4 gm. of food during the consecutive days following the change of diet. Had the mushroom-containing diet proven merely unpalatable the animals would probably have eaten less than 8 gm. during the first day on the diet. A more plausible explanation might include an adverse gastro-intestinal reaction to the diet, followed by a diminished appetite for mushroom-containing food.

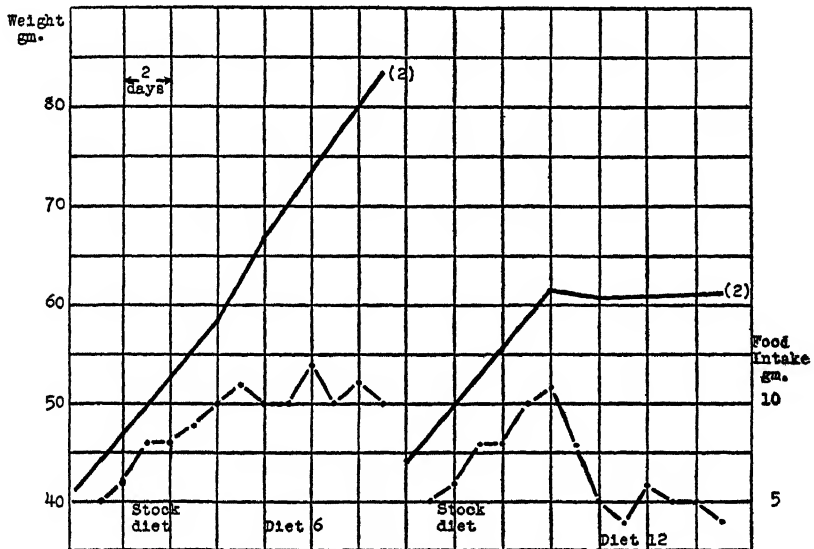


Fig. 5 Simultaneous food consumption and growth curves during transition from the stock diet to yeast and mushroom diets.

An attempt was then made to study the motor activity of the alimentary tract after consumption of mushroom and yeast diets. Six 200-gm. rats which were receiving the stock colony diet were starved for 36 hours. Each was then given 6 gm. of food, three receiving diet 8 and the three others diet 12. After 1½, 3 and 5 hours one animal on each diet was killed, the contents of stomach and upper three-quarters of the small intestine removed, dried and weighed. No significant differences were found. After 5 hours 20 to 25 per cent of the meal still remained in the stomach in both cases; the quantity found in the intestine was constant and of small magnitude.

## DISCUSSION

From the preceding data it follows that a study of the nutritive properties of mushrooms with rats is beset with difficulties, of which the subnormal consumption of the ration is outstanding. This latter effect was especially marked when mushrooms were included in the diet from the beginning of an experiment. It is well exemplified by the effect of even a low percentage of mushrooms in an otherwise satisfactory diet. The introduction of only 10 per cent of mushrooms into diet 8 reduced both food intake and growth by 40 to 45 per cent. The explanation of this effect has not been found. The likelihood that mushroom diets are merely unpalatable is opposed by a considerable amount of evidence which has been discussed previously. Observations tending to exclude the possibility that a toxic factor is involved have likewise been pointed out in previous discussion. Lack of digestibility could hardly be advanced as an explanation, especially when low levels of mushroom intake are involved. Further evidence opposing such a view is the low fiber content (Skinner, Peterson and Steenbock, '33), the high digestibility of the nitrogen-containing fraction (equivalent to 30 per cent or more of the whole if considered as protein), and the high content of water-soluble constituents. The belief that mushrooms are of such poor nutritional value that they serve to dilute the ration can hardly be upheld; evidence shown in equalized intake experiments with different diets is quite to the contrary. Failure to find gastro-intestinal irritation or gaseous accumulation in autopsied animals would tend to eliminate the possibility of such disturbances as causes of diminished food consumption.

In the studies of the vitamin B content of mushrooms considerable evidence has indicated that some factor of the vitamin B complex, other than B or G is lacking, or present in only small quantity. This factor would seem to be heat stable and closely associated with vitamin B. However, this evidence cannot be accepted as final since the reactions may be clouded by the effects of the poor consumption of mushroom diets.

A study of the mushroom protein becomes involved in the same difficulties. The complete failure of growth and the short survival period of animals receiving mushrooms as their only protein source, when compared with the effect of the addition of casein to the same diet, would suggest a deficiency in the mushroom protein. However, the experiments with the water-soluble fraction of the mushrooms lend no encouragement to a further study of the protein fraction. Better consumption and growth-promoting properties were shown with a 40 per cent level of the water-soluble fraction than with an equivalent amount of the whole mushroom. Most of the protein, as well as the factor which is responsible for poor food consumption, would likely appear in the insoluble material. Although no studies have been made with this insoluble fraction it is probable that it, like the whole mushrooms, would not be consumed in satisfactory quantities.

It is apparent from our studies with rats that the mushroom does not serve as a particularly valuable dietary constituent. It is a fair source of vitamin B and ranks favorably with milk and yeast as a carrier of vitamin G. Limited data tend to show that the protein is incomplete. While it is shown that subnormal food consumption and growth follow the introduction of as little as 10 per cent mushrooms into an otherwise satisfactory diet, there is no evidence that this effect is due to toxicity.

It is unlikely that these findings are of significance in practical human nutrition. In the great majority of cases mushrooms constitute only a small part of an occasional meal and are included in the human dietary largely because of their 'condimental value' rather than for their nutritive properties.

#### SUMMARY

The nutritive properties of the mushroom *Agaricus campestris* have been studied with albino rats.

Diets which contained mushrooms were consumed in subnormal quantities, and consequently growth was subnormal on these diets. Exceptions to this general result were ob-



served when mushrooms were fed to animals which were depleted in vitamin B or G.

Mushrooms were found to be a relatively good source of vitamins B and G. Levels of 10 per cent and 5 per cent of the diet on a dry weight basis supplied sufficient B and G, respectively, to support satisfactory growth.

The data indicate that a diet containing 10 per cent of mushrooms as the only source of vitamin B is deficient in some factor other than B or G.

A preliminary study has indicated that mushroom protein is incomplete.

A detailed investigation of these deficiencies is hampered by the poor consumption of diets which contain mushrooms.

There is no evidence to show that *Agaricus campestris* contains a toxic principle.

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# THE EFFECT OF THE INGESTION OF SALINE WATERS UPON THE pH OF THE INTESTINAL TRACT, THE NITROGEN-BALANCE AND THE COEFFICIENT OF DIGESTIBILITY

V. G. HELLER, J. R. OWEN AND LUCILE PORTWOOD

*Department of Agricultural Chemistry Research, Oklahoma Experiment Station,  
Oklahoma Agricultural and Mechanical College, Stillwater, Oklahoma*

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In previous articles from this laboratory (Heller, '32 and '33; Heller and Paul, '34) attention has been called to the occurrence of saline and alkaline waters over large areas of the earth's surface, and to the injurious effect of these waters upon domestic animals when used in excessive concentrations for drinking purposes. While rather specific concentrations have been established as the maximum amount permitting growth, reproduction, and general well-being of animals no definite cause has yet been determined as being responsible for the deaths and injuries noted although possible reasons were offered in the articles cited.

It has frequently been suggested that the ingestion of large quantities of salts and alkalies would seriously alter the pH of the stomach and intestinal tract through their buffering action and possibly poor assimilation might result in the inability of an animal to gain weight under these conditions. A series of tests have been carried out in which the coefficients of digestibilities and nitrogen-balances were determined to ascertain if osmotic pressure, assimilation, or secretion might be altered and the poor response to food intake be due to poor absorption of the nutrients. In a similar series of tests the pH of the entire intestinal tract has been determined at stated intervals comparing rats receiving distilled water with others

receiving varying concentrations of different salt solutions. This work has been in progress for several years and the results obtained are briefly summarized in this article.

#### EXPERIMENTAL

Rats, chickens and hogs have been used in these tests. Rats were used far more extensively, and all data recorded will be for this species excepting that for hogs shown in table 3. The animals used were of vigorous stock from our colony. The basic ration which had been used for years, consisted of mixed cereals amply supplemented with animal proteins, minerals and vitamins. The metabolism cages used were of our own design which have frequently been improved during the past 15 years. Though no cages used for the rats represent perfect recovery, this model approaches an ideal.

In all of the tests, groups of comparable rats were divided into similar groups of four or more and placed in round metal cages. The basic ration was fed ad libitum. The drinking solutions were prepared by dissolving given amounts of pure salts in distilled water in the amounts indicated and given to the animals in inverted vacuum fountains. In every case the growth of the individual rats, the amount of solution drunk daily, as well as the weight of the food consumed were recorded. The animals were kept under constant observation from 4 to 10 weeks previous to the digestion tests. They were then placed in the metabolism cages at least 3 days prior to the time samples were taken to permit the animals to adjust themselves to their new environment. During the time the tests were in progress, the exact amount of food eaten and the drinking solution consumed were recorded daily. The feces were collected several times daily and quickly dried. The urine was preserved with  $\text{H}_2\text{SO}_4$  and made up to definite volume. The feed, feces and urine were analyzed by the usual accepted methods (condensed statements of which may be found in Peters and Van Slyke, '31) and the nitrogen-balances and digestibilities calculated and recorded. The trials have been repeated with different sets of rats and for

different years. Figures presented represent the average of 24 rat days in most cases.

Table 1 represents the averages of several hundred trials with young mature rats. The feed for each group being similar, but the drinking waters varied from distilled water in one case, to various salt solutions in different concentrations as indicated. These tests were conducted during the winter months.

TABLE 1

*The nitrogen-balance and the apparent coefficient of digestibility of rats receiving saline drinking waters*

DRINKING WATER	NITROGEN BALANCE (MILLIGRAMS PER PERIOD)	APPARENT COEFFICIENT OF DIGESTIBILITY				
		Protein	Ash	Fat	Fiber	Nitrogen free extract
Distilled water	+ 47.3	62.3	28.3	81.6	15.2	82.3
NaCl 1 per cent	+ 34.5	71.5	53.2	87.3	17.8	80.6
NaCl 1.5 per cent	+ 32.5	75.2	68.9	85.6	28.1	81.1
NaCl 2 per cent	+ 30.5	75.2	76.5	81.8	27.1	81.5
MgSO <sub>4</sub> 1 per cent	+ 34.5	71.4	42.9	85.6	20.6	79.7
MgSO <sub>4</sub> 1.5 per cent	+ 40.6	73.3	59.1	87.2	26.6	79.5
MgSO <sub>4</sub> 2 per cent	+ 30.4	65.5	58.0	79.4	17.7	80.1
CaCl <sub>2</sub> 1 per cent	+ 42.5	71.8	41.4	84.1	25.3	80.1
CaCl <sub>2</sub> 1.5 per cent	+ 40.0	72.6	47.6	85.9	21.9	77.9
CaCl <sub>2</sub> 2 per cent	+ 41.1	66.7	51.6	76.3	28.5	80.1

Table 2 represents a similar series of results using younger animals during the spring months. The only similarity between the two groups being the manner of conducting the experiment.

TABLE 2

*Apparent coefficient of digestibility and nitrogen-balance of rats consuming basic ration and drinking saline water*

WATER CONSUMED	NITROGEN BALANCE (MILLIGRAMS PER PERIOD)	APPARENT COEFFICIENT OF DIGESTIBILITY				
		Protein	Ash	Fat	Fiber	Nitrogen free extract
Distilled water	+ 17.1	76.8	38.6	77.9	16.7	88.5
Saturated Ca(OH) <sub>2</sub>	+ 16.6	77.6	28.4	79.2	16.6	85.7
Saturated CaSO <sub>4</sub>	+ 25.3	76.6	32.9	78.2	18.2	86.8
1.5 per cent CaCl <sub>2</sub>	+ 22.2	76.3	24.0	82.1	20.7	87.7
1.5 per cent MgSO <sub>4</sub>	+ 24.3	78.9	29.9	84.1	21.4	86.0

Table 3 records similar data, in this case for hogs.

TABLE 3

*Apparent coefficient of digestibility and nitrogen-balance of hogs drinking distilled and salty waters*

WATER CONSUMED	NITROGEN BALANCE (MILLIGRAMS PER PERIOD)	APPARENT COEFFICIENT OF DIGESTIBILITY				
		Protein	Ash	Fat	Fiber	Nitrogen free extract
Normal	+ 42.7	71.7	28.7	59.5	15.8	74.3
1 per cent NaCl	+ 48.5	81.5	56.8	73.4	42.3	86.1

Table 4 records similar data for animals receiving distilled water and having salts added to the basic ration. In this case distilled water was given ad libitum to all groups. The water consumption of the salt-eaters was much increased, evidently in an attempt to flush the salts from the body.

TABLE 4

*Apparent coefficient of digestibility and nitrogen-balance of rats receiving varying amounts of salts added to basic ration*

SALT ADDED TO BASIC RATION	NITROGEN BALANCE (MILLIGRAMS PER PERIOD)	APPARENT COEFFICIENT OF DIGESTIBILITY				
		Protein	Ash	Fat	Fiber	Nitrogen free extract
None	+ 29.7	73.1	50.6	76.5	19.4	81.8
5 per cent NaCl	+ 20.9	80.2	65.7	85.1	18.7	81.0
3 per cent CaCl <sub>2</sub>	+ 18.4	72.6	48.6	84.2	15.1	80.8
5 per cent Na <sub>2</sub> HPO <sub>4</sub>	+ 26.5	75.2	60.8	85.5	12.6	83.2

Possibly the figures for ash should have been omitted from the standpoint of true digestibility, but they have been included as showing the trends produced by various ions.

An inspection of the data presents one fact which is contrary to what one would anticipate and that is that the maximum amount of salts that can be consumed without producing serious results is evidently not responsible for any interference of food utilization; in fact, there is considerable evidence that the presence of these salts in quantities less than the amount which produces serious disturbances, such as rapid loss of weight, inability to produce and nourish young and

even death, facilitates digestion and absorption. The concentrations of soluble salts producing the injuries referred to have been reported elsewhere as approximately 15,000 parts per million.

#### pH OF INTESTINAL TRACT

After the digestion trials with the animals used in the above tests were completed, the animals were then sacrificed to make a study of the effect of these salts upon the hydrogen ion concentration of the digestive tract. These animals were guillotined and samples removed from designated portions of the entire length of the intestinal tract.

The method of determining the hydrogen ion concentration of a material like feces is always debatable. Many workers have used colorimetric methods but with the large amount of buffer material present it is doubtful if it is sufficiently accurate. The electrometric, the quinhydrone and the glass electrode have been recommended and conducted by various workers. When this work was started the procedure of Redman, Willimott and Wokes ('27) seemed to offer greater advantages. At about this time, Eastman and Miller ('32, '35) reported the results of the effect of diet on the pH of the rats' intestinal contents using the glass electrode. The method used in this laboratory was one devised for our own work. The method of sampling, the amount of dilution, and the time of shaking being chosen as most favorable for these determinations. The method is briefly outlined as follows:

The intestine was opened and a sample of the contents removed with a small glass scoop, deposited in a dry test tube and 5 cc. of conductivity water added. The mixture was then macerated and 10 drops of an acetone solution of quinhydrone containing 0.036 gm. per cubic centimeter were added. A portion of the mixture was then transferred to a Bailey electrode and shaken for 3 minutes; attached to the potentiometer, and read exactly 3 minutes later. The variations in the readings produced by the amounts of material were of much less importance than the matter of time and temperature. The re-



sults of several hundred such determinations have been collected, averaged and condensed in the following table.

TABLE 5

*pH of the contents of the intestinal tract of rats receiving salts and acids added to food*

RATION	STOMACH	DUODENUM	JEJUNUM	ILEUM	CECUM	DESCENDING COLON
Basic A	4.82	6.55	7.27	6.94	6.95	7.26
Basic A + 3 per cent $\text{CaCO}_3$	5.38	6.26	7.86	7.39	7.37	7.49
Basic A + 5 per cent $\text{CaCO}_3$	4.66	6.07	7.19	7.10	7.19	7.29
Basic A + 3 per cent $\text{CaCl}_2$	4.01	6.55	7.92	7.28	7.36	7.52
Basic A + 4 per cent $\text{CaCl}_2$	3.11	6.37	7.39	7.05	7.20	7.25
Basic A + 5 per cent $\text{NaH}_2\text{PO}_4$	4.60	6.08	6.61	6.69	6.78	6.71
Basic A + 3 per cent citric acid	4.09	6.32	7.75	7.32	7.24	7.47
Basic A + 5 per cent citric acid	3.10	5.89	7.07	7.22	7.09	7.13

An inspection of the data for these rats, which were from 5 to 6 months of age, does not indicate that the intestinal content is appreciably altered by the presence of various acid and alkaline salts in the drinking supply. Calcium chloride tends to increase the acidity of the stomach but increases the alkalinity of the lower intestine.

The fact that the pH is only slightly altered by salts confirms the conclusion drawn by Eastman and Miller ('35) in regard to the effect of various diets, namely, that in most cases the food causes little changes in the pH. Possibly this statement would not hold true for animals receiving a more concentrated drinking solution. The problem is involved in that at higher concentrations the animals die and the data cannot be obtained.

## CONCLUSIONS

The use of drinking waters containing considerable quantities of dissolved salts does not interfere with the nitrogen utilization of normal rations. Likewise the apparent coefficients of digestibility of the constituents of the ration are not interfered with; in fact, the trend of all determinations indicate that assimilation or digestibility is aided by the presence of reasonable amounts of salt so long as the total content is kept below the concentration where serious disturbances in growth and reproduction takes place.

The pH of the entire intestinal tract is not appreciably altered by the presence of alkali or acid salts in the drinking water, providing the concentration present does not exceed the amount permitting a somewhat normal life. Observed changes are more apparent in the stomach than in the lower intestine.

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# THE DIFFERENTIAL ANTIRACHITIC ACTIVITY OF VITAMIN D MILKS<sup>1</sup>

ROBERT W. HAMAN AND HARRY STEENBOCK

*Department of Agricultural Chemistry, University of Wisconsin, Madison*

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The recent commercial acceptance of vitamin D milks, whether produced by feeding cows irradiated yeast,<sup>2</sup> by direct irradiation,<sup>2</sup> or by the addition of vitamin D concentrates, has resulted in the appearance of a considerable literature discussing their comparative antirachitic effectiveness in relation to each other and to irradiated ergosterol and cod liver oil. The stimulus for this discussion growing out of the report of Massengale and Nussmeier ('30) working with chicks, was provided by the clinical reports of Hess, Lewis and Rivkin ('30), working with infants and confirmed by De Sanctis and Craig ('30) and Barnes and Brady ('30), that the vitamin D from irradiated ergosterol was not as effective as that from cod liver oil when compared on a rat unit basis.

The majority of clinicians who have investigated the antirachitic effectiveness of vitamin D milks are of the opinion, 1) that they are very effective both for the prevention and cure of rickets when used in proper amounts, 2) that they are more efficient unit for unit than other antirachitics, and 3) that the various milks compared with each other are equally effective. Hess, Lewis, MacLeod and Thomas ('31) fed milk

<sup>1</sup>Published with the permission of the director of the Wisconsin Agricultural Experiment Station.

A preliminary report of the data in this paper was presented before the American Institute of Nutrition, April 10, 1935. Similar data were presented by Bethke, Krauss, Record and Wilder, Proc. Am. Inst. Nutrition, J. Nutrition, vol. 9, suppl. p. 7, 1935.

<sup>2</sup>Referred to hereafter as 'yeast milk,' or 'irradiated milk.'

produced by feeding cows irradiated ergosterol and irradiated yeast to 102 infants. Both rachitic and non-rachitic infants were included in their experiments. The authors report that the lower level, 60 units<sup>3</sup> daily did not successfully prevent or cure rickets. However, the higher level, 120 units daily, was successful in both their preventive and curative studies. Wyman and Butler ('32) reported that yeast milk containing 160 units per quart at daily levels of 160 and 200 units initiated calcium deposition in the bones of rachitic infants within 2 weeks of the beginning of treatment. Four infants were used in this study. Hess and Lewis ('32) concluded from a study including ninety-eight infants that irradiated milk is highly effective both for the prevention and cure of rickets. The daily intake of vitamin D was 37 and 50 units. Mitchell, Eiman, Whipple and Stokes ('32) fed irradiated milk and milk from irradiated cows to thirty-four infants. The irradiated milk contained 65 units per quart and the milk from irradiated cows 22 units per quart. Both milks were reported as being successful antirachitics. Gerstenberger and Horesh ('32) fed two rachitic infants milk from cows that were fed irradiated ergosterol. The daily intake of vitamin D for the infants was 80 units. The authors concluded that satisfactory progress in healing occurred in 10 to 11 weeks. The healing was not complete at this time. Hess and Lewis ('33) in a curative study fed irradiated milk at daily levels of 28 and 42 units, and yeast milk at 60 and 90 units. They concluded that 35 to 42 units daily of irradiated milk and 70 units or more of yeast milk were sufficient for protection against rickets. The efficacy ratio of the two milks was reported as approximately 1:2. Kramer and Gittleman ('33) fed in a curative type of experiment both irradiated milk and yeast milk at daily levels for each milk of 40 and 50 units. Ten rachitic infants were included in this study. The authors could not demonstrate any significant difference between the two milks. They concluded, because of the definite curative effect of a daily intake

<sup>3</sup>The vitamin D potencies are expressed as Steenbock units. For all practical purposes we have found that 1 Steenbock unit is equivalent to  $3\frac{1}{2}$  International units.

of 40 units that vitamin D milk is a very effective antirachitic agent. Drake, Tisdall and Brown ('34) studied the antirachitic effectiveness of cod liver oil, Viosterol, and irradiated milk in a preventive study with 141 infants. No attempt was made to compare the effectiveness of these antirachitics. They reported 35 to 70 units of irradiated milk daily were sufficient for the prevention of the development of either moderate or marked rickets. Jeans and Stearns ('34) concluded from a study with seven infants that irradiated milk, cod liver oil concentrate milk, and cod liver oil and milk separately, are all equivalent upon a rat unit basis. Furthermore, that 50 units per day were insufficient for the best development of the infant although rickets may be prevented. They used as criteria, roentgenograms, calcium retention, serum Ca and P determinations, and rates of growth. Fifty units daily was the maximum vitamin D intake and at no time during the experiment did an infant develop rickets. Wyman, Eley, Bunker and Harris ('35) in a curative study fed vitamin D from irradiated milk and yeast milk at daily levels of 30 to 50, and 50 to 65 units, respectively. They used roentgenograms and the rate of increase of the  $\text{Ca} \times \text{P}$  product as criteria. They concluded that the two milks unit for unit were equivalent and that vitamin D milk containing 50 units per quart will produce demonstrable healing in infants in 4 weeks.

Gerstenberger et al. ('35) in a carefully controlled curative study fed irradiated milk and yeast milk to thirteen rachitic infants. They used roentgenograms and serum Ca and P determinations as criteria. They reported that 40 units of vitamin D milk produced satisfactory changes in the bones in 10 to 12 weeks and in the blood in 49 to 62 days. In regard to the comparative effect of the two milks, they concluded that there were no practical differences although if a slight difference existed it was in favor of irradiated milk. Barnes, in two publications ('33), ('34), has reported the results of two series of experiments wherein cod liver oil concentrate milk was fed both to rachitic and non-rachitic infants. In his

first paper ('33) he reported the results of his studies with fifteen rachitic infants. They were fed vitamin D concentrate milk at daily levels of 100 and 150 units. He reported that all of the infants responded promptly. In his second paper ('34) he reported that thirty-two normal infants were protected against rickets when fed 50 units daily of cod liver oil concentrate milk. Six infants that were slightly rachitic at the beginning of the experiment showed satisfactory healing. A control group of twenty-five infants showed an incidence of rickets of 56 per cent. Roentgenograms and gain in weight were used as criteria. It was concluded that the vitamin D of irradiated milk and of cod liver oil concentrate added to milk, are equally efficient. Wilson ('34) fed cod liver oil concentrate milk in a preventive study to thirty-three infants. The daily intake of vitamin D was 90 to 140 units. The criteria consisted of monthly roentgenograms and body weights and lengths. Of the thirty-three infants, fourteen remained normal, seventeen developed slight rickets, and two a moderate degree of rickets. The authors state in the conclusion of their report that the number of units of vitamin D concentrate per quart that are necessary can be determined only by further study. Rapoport, Stokes and Whipple ('35) in both a curative and preventive study with twenty-three male Negro infants reported that irradiated evaporated milk containing 46 units per 14.5-ounce can was adequate for the prevention of rickets. They also reported that this milk was inadequate for the cure of rickets in Negro infants. Roentgenograms, gain in weight, and increase in body length were used as criteria.

The reason for the apparent greater efficiency of vitamin D milks over other sources of vitamin D has not been determined. Ansbacher and Supplee ('34) have postulated that the vitamin may be combined with a protein fraction, thus enhancing its absorption and utilization. Very recently Lewis ('35) has reported that propylene glycol as a solvent for vitamin D greatly increased its effectiveness as compared with corn oil as a solvent when added to milk and included in the

dietary of rachitic infants. Waddell ('34) believes that the high efficiency of irradiated milk can be explained on the basis that it contains the activated provitamin of crude cholesterol, which he found to be so effective with chicks and which has been shown recently by Tisdall, Drake and Brown ('35) to be as effective as cod liver oil for infants. The reported high efficiency of yeast milk, however, cannot be explained upon this basis.

Krauss, Bethke and Monroe ('32) working with chicks found that "on a rat unit basis it required four times as many vitamin D units in yeast milk butter fat as in cod liver oil to bring about the same degree of calcification." Supplee, Bender and Dorcas ('32) working with chicks found irradiated milk to be an effective agent for the prevention of rickets. However, these investigators made no attempt to compare the effectiveness of irradiated milk with any other antirachitic agent.

In our studies we have availed ourselves of the striking parallelism that exists between the infant and the chick in the directional response to vitamin D therapeusis to make a quantitative comparison of the efficiency of an equal number of units of vitamin D from irradiated milk, yeast milk, irradiated ergosterol, and irradiated cholesterol.

Briefly our data show that yeast milk is far less effective than irradiated milk for the chick; that irradiated milk, irradiated cholesterol, and cod liver oil are of approximately the same order of effectiveness; and that the constituents of milk as a vehicle for vitamin D do not increase its effectiveness.

#### EXPERIMENTAL AND RESULTS

Day-old White Leghorn chicks, obtained from commercial hatcheries, were placed in groups of fifteen in brooders with wire screen bottoms. They were given a rachitogenic diet (Hart, Kline and Keenan, '31) with additions of various amounts of vitamin D. At the end of 5 weeks they were killed, both tibia were removed and analyzed for ash after extraction with alcohol.



In this type of experiment to obtain results that are significant, it is necessary to feed levels of vitamin D that will produce a rickets of moderate or slight severity. To insure securing the appropriate levels of intake of vitamin D, preliminary assays using the line test were carried out with five rats at each level of intake and in most instances prophylactic assays using at least nine animals at each level of intake were carried out as well.

In our first series we determined the comparative antirachitic effectiveness of 'irradiated milk,' 'yeast milk,' irradiated ergosterol, and cod liver oil. For these determinations the cod liver oil and irradiated ergosterol were diluted with Wesson oil so that the desired level of intake of vitamin D was obtained when the oil was added to the extent of 1 per cent of the diet. The 'irradiated milk' and 'yeast milk' were diluted with untreated milk so that the effective intake of vitamin D was obtained with the consumption of 90 cc. of milk daily. This amount was thoroughly mixed into 97 gm. of the basal ration. For control purposes untreated milk was included in the rations of the cod liver oil and irradiated ergosterol groups in the same proportion. The rations were fed to the chicks twice daily.

The results of this series presented in table 1 reveal the relative inefficiency of yeast milk as compared with both cod liver oil and irradiated milk. Ten units of vitamin D from yeast milk produced no evidence of protection, whereas 2.5 units given either as cod liver oil or as irradiated milk revealed a demonstrable effect.

The data in table 1 also show that apparently irradiated milk was not so effective as cod liver oil. However, the differences are small and well within the range of experimental error as the unitage administered had been determined only by means of the line test on five animals.

That irradiated milk has a high efficiency per unit of vitamin D in clinical tests has already been discussed. On the supposition that the high efficiency might be due, at least in part, to the favorable effect of the constituents of milk other

than the vitamin prompted us to feed milk to all of our groups in this experiment. The results suggest that irradiated milk has no greater efficiency per unit than cod liver oil.

A second series of experiments was carried out in an attempt to confirm our first results and to determine the factors responsible for the greater effectiveness of a unit of vitamin D in irradiated milk than in 'yeast milk.' We investigated the possibility that this difference could be assigned to either the skimmed milk or the butter fat.

TABLE 1

*Series I. Ash in the tibia of chicks fed vitamin D from cod liver oil, irradiated ergosterol, irradiated milk and yeast milk*

SUPPLEMENTS TO THE BASAL RATION	STEENBOOK UNITS PER 100 GM. RATION	NUMBER OF CHICKS	AVERAGE WEIGHT OF CHICKS	AVERAGE WEIGHT OF ASH	AVERAGE ASH
			<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
Untreated milk	0	13	166	0.2009	31.73
Untreated milk + cod liver oil	2.5	13	191	0.2217	36.08
Irradiated milk	2.5	15	193	0.2215	33.69
Yeast milk	2.5	12	145	0.1692	32.33
Untreated milk + cod liver oil	5.0	13	212	0.2570	38.35
Irradiated milk	5.0	10	232	0.2981	36.26
Yeast milk	5.0	13	161	0.2025	32.72
Yeast milk	10.0	14	156	0.1693	32.60
Untreated milk + irradiated ergosterol	12.5	13	182	0.1961	32.93
Untreated milk + irradiated ergosterol	50.0	13	223	0.2672	37.55
Untreated milk + irradiated ergosterol	100.0	13	217	0.3433	44.57

For this purpose cream was separated, respectively, from yeast milk, irradiated milk and untreated milk. The cream was churned to butter and the butter fat was melted, decanted, and filtered. The skimmed milk was preserved by freezing. Both the curative line test method and the prophylactic method with analysis for bone ash were used to assay the butter fats. The line test was used for preliminary results and the prophylactic test with ash analyses for the final values.

In the chick rations the amount of butter fat and skimmed milk was kept constant. Every 2 kg. of ration contained 80

gm. of butter fat. Immediately before feeding 100 cc. of skimmed milk were added to each 100 gm. of ration. To attain this constancy, dilutions with untreated skimmed milk and untreated milk butter fat were obviously necessary. To determine the influence of the skimmed milk fraction the experimental groups were arranged so that the skimmed irradiated milk was fed with butter fat from yeast milk and

TABLE 2

*Series II. Ash in the tibia of chicks fed vitamin D from cod liver oil, irradiated milk butter fat and yeast milk butter fat, together with a constant amount of skimmed milk*

SUPPLEMENTS TO THE BASAL RATION	STEENBOCK UNITS PER 100 GM. RATION <sup>1</sup>	NUMBER OF CHICKS	AVERAGE WEIGHT OF CHICKS	AVERAGE WEIGHT OF ASH	AVERAGE ASH
			gm.	gm.	per cent
Untreated milk butter fat + untreated skimmed milk	0	13	204	0.2152	33.92
Cod liver oil + untreated milk butter fat + untreated skimmed milk	2.5	13	231	0.2532	37.72
Irradiated milk butter fat + untreated skimmed milk	2.0	14	247	0.2671	35.22
Yeast milk butter fat + untreated skimmed milk	3.6	14	196	0.2020	32.61
Cod liver oil + untreated milk butter fat + untreated skimmed milk	5.0	12	251	0.2884	42.11
Irradiated milk butter fat + untreated skimmed milk	4.0	12	258	0.3231	39.62
Yeast milk butter fat + untreated skimmed milk	7.25	11	201	0.2418	34.28
Irradiated milk butter fat + yeast skimmed milk	4.0	14	265	0.3340	40.60
Yeast milk butter fat + irradiated skimmed milk	7.25	11	194	0.1948	32.28

<sup>1</sup> Unitage determined by prophylactic assays with rats.

vice versa. In other groups untreated skimmed milk was fed with both butter fats. Skimmed milk and butter fat from untreated milk were added to the rations of both the negative and positive control groups.

The data presented in table 2 reveal that the yeast milk butter fat afforded no protection at the levels fed. The addition of skimmed irradiated milk or skimmed control milk

did not increase its effectiveness. The irradiated milk butter fat, regardless of the skimmed milk supplement afforded approximately the same degree of protection as cod liver oil.

The results of this series therefore confirmed our previous findings in regard to the difference in action of the vitamin D from the two milks, and furthermore, assigned this difference to the butter fat fraction of the milk.

We felt, however, that it was desirable to secure confirmatory evidence on a more direct quantitative basis. To this end we ran a third series of experiments. Since in series 2 the skimmed milk portion was without effect, we decided to use only the butter fat from the various milks as the vitamin D supplements to the rations. The feeding of butter fat as the only supplement had the advantage of eliminating the repeated standardization made desirable by possible daily variations in the vitamin D content of the whole milk.

The butter fats that were used in this third series were prepared in the same manner as those used in series 2. The butter fat additions, 108 gm. in 2 kg. of ration, were adjusted by dilution with control butter fat so that equal amounts of fat were added to each ration. The general plan of this series as well as the results are shown in table 3.

The chicks on this series of experiments had to be started before the assays on the butter fats had been completed and unfortunately, the actual potencies were not as anticipated. It was found from our prophylactic assays with rats that the irradiated milk butter fat was approximately 35 per cent more potent than calculated, whereas the yeast milk butter fat was about 74 per cent as potent. In spite of this we can conclude from the results of this experiment, table 3, taking into account the results of our rat assays, that the data of this series confirm the data obtained in our previous experiments. The irradiated milk butter fat was found to be as efficient as cod liver oil whereas the yeast milk butter fat was very inefficient. Ten rat units per 100 gm. of ration did not produce any evidence of protection.

In a fourth series of experiments we increased the level of yeast milk butter fat to 25 units per 100 gm. of basal ration in the hope that a demonstrable healing would be produced. This necessitated a high level of butter fat in the ration, viz., 12.5 per cent. However, this amount of butter fat inhibited normal growth during the early part of the experiment. We consequently analyzed the bones of one-half of the chicks in the group at 6 weeks instead of at 5 weeks which was our customary procedure.

TABLE 3

*Series III. Ash in the tibia of chicks fed vitamin D from cod liver oil, irradiated milk butter fat and yeast milk butter fat*

SUPPLEMENTS TO THE BASAL RATION	STEENBOCK UNITS PER 100 GM. RATION <sup>1</sup>	NUMBER OF CHICKS	AVERAGE WEIGHT OF CHICKS	AVERAGE WEIGHT OF ASH	AVERAGE ASH
			<i>gm.</i>	<i>gm.</i>	<i>per cent</i>
Untreated milk butter fat	0	14	194	0.2041	32.71
Cod liver oil	2.5	11	200	0.2417	35.86
Irradiated milk butter fat	3.4	14	241	0.3204	42.92
Irradiated milk butter fat	5.0	15	253	0.3541	45.53
Cod liver oil	5.0	15	240	0.3098	39.51
Irradiated milk butter fat	6.75	11	283	0.4390	46.21
Irradiated milk butter fat	8.4	14	271	0.4003	46.94
Irradiated milk butter fat	10.1	13	237	0.3639	46.58
Yeast milk butter fat	2.5	14	217	0.2295	34.27
Yeast milk butter fat	5.0	12	185	0.2056	34.11
Yeast milk butter fat	10.0	12	201	0.2133	33.63

<sup>1</sup> Unitage determined by prophylactic assays with rats.

We were interested further in studying the efficacy of irradiated cholesterol as reported by Waddell ('34). For this study a cholesterol preparation was prepared and irradiated according to his technic. Preliminary assays with rats using the 10-day line test method revealed that 0.375 mg. of the irradiated cholesterol was equal to 1 rat unit. The irradiated ergosterol was similarly assayed. Both of these preparations were dissolved in Wesson oil and the desired level was obtained when the oils were added to the extent of 2 per cent of the ration. Irradiated milk butter fat was also included in this series. The results are included in table 4.

From the data presented it is apparent that the vitamin D in irradiated milk butter fat is at least ten times more effective than the vitamin D in yeast milk butter fat. Irradiated cholesterol was found to be equally as effective as irradiated milk butter fat and approximately twenty times more effective than irradiated ergosterol. We have therefore confirmed the relative high efficiency of irradiated cholesterol for the chick as reported by Waddell.

TABLE 4

*Series IV. Ash in the tibia of chicks fed vitamin D from irradiated milk butter fat, 'yeast milk' butter fat, irradiated cholesterol and irradiated ergosterol*

SUPPLEMENTS TO BASAL RATION	STEENBOOK UNITS PER 100 GM. RATION <sup>2</sup>	NUMBER OF CHICKS	AVERAGE WEIGHT OF CHICKS	AVERAGE WEIGHT OF ASH	AVERAGE ASH
No additions	0	14	gm. 185	gm. 0.1764	per cent 33.47
Irradiated cholesterol	2.5	15	234	0.2616	40.70
Irradiated milk butter fat	3.4	15	247	0.2659	42.92
Irradiated cholesterol	5.0	12	286	0.3987	46.49
Yeast milk butter fat	25	7	184	0.2003	40.27
Yeast milk butter fat <sup>4</sup>	25	7	233	0.2761	36.21
Irradiated ergosterol	50	14	260	0.2983	41.40

<sup>1</sup> Continued on experiment for 6 weeks instead of 5.

<sup>2</sup> Unitage determined by prophylactic assays with rats.

#### DISCUSSION

The results that we have presented are for the most part not in agreement with the clinical reports that have appeared in the literature. This apparent lack of agreement may or may not be real. The efficacy ratio of cod liver oil to irradiated ergosterol for the chick has been shown to be approximately 1:20 and for the infant, 1:4 (Hess and Lewis, '33). Obviously the chick is the more sensitive of the two in regard to its differential response to vitamin D from these two sources. Since our results revealed that the efficacy ratio of irradiated milk to yeast milk is approximately 1:10, and since from the above the chick is approximately five times as sensitive to a unit of vitamin D from cod liver oil and irradiated ergosterol, it is very probable that a twofold difference in effectiveness of

the two milks is the most that could be expected for the infant. Hess and Lewis ('33) have reported this ratio of effectiveness of the two milks, although most investigators are agreed that the two milks are of equal effectiveness. From our experience with biological determinations of this nature we have become more and more impressed with the difficulties associated with the accurate determination of differences in vitamin D activity. These difficulties obviously tend, out of circumstance, to be magnified in clinical studies.

In our experiments we have been unable to confirm the reported high efficiency of yeast milk and irradiated milk as contrasted with cod liver oil or irradiated ergosterol. Nor have we obtained any indication that the presence of the constituents of milk as a vehicle for the vitamin D of either cod liver oil or irradiated ergosterol influenced either utilization or effectiveness. In series II we investigated the possibility that the skimmed milk fraction might have an influence on the antirachitic activity of these milks. Our results revealed that the skimmed milk fraction was without effect and furthermore that the differences in regard to the effectiveness of the two milks observed in our first series could be assigned entirely to the butter fat fraction.

Our results therefore indicate that the quantitative relation which has been found to exist in the response of the chick and the infant to cod liver oil and irradiated ergosterol does not exist with vitamin D milks. Our data although obtained with the chick reveal the necessity for more clinical studies of a quantitative nature concerning the antirachitic effectiveness of vitamin D milks in relation to each other and to other antirachitic agents.

#### SUMMARY

Briefly, the results of our experiments show that for the chick and per unit of vitamin D:

1. Irradiated milk, cod liver oil, and irradiated cholesterol are of approximately the same order of effectiveness.

2. Yeast milk is approximately one-tenth as effective as irradiated milk. This difference was confined to the respective butter fat fractions and was not influenced by the skimmed milk fraction.

3. The constituents of milk as a vehicle for vitamin D do not influence its effectiveness.

4. Our experiments give no support to the possibility that the baby chick could be used to greater effectiveness than the rat for ascertaining the degree of antirachitic effectiveness of different vitamins D for the human.

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## STUDIES ON GROWTH

### III. B AND G AVITAMINOSIS IN CECECTOMIZED RATS <sup>1</sup>

WENDELL H. GRIFFITH

*Department of Biological Chemistry, St. Louis University School of Medicine  
St. Louis*

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It is now well established that young rats survive the lack of vitamin G for much longer periods than those deprived of vitamin B. In complete B deficiency polyneuritic signs appear and death occurs after 20 to 50 days, whereas in G deficiency rats may survive for 50 to 100 days or more. Since growth ceases within 5 to 10 days after being placed on either the B-free or G-free diet, the long survival of animals deprived of G becomes a matter of considerable interest. Certainly the supply of G which is available for maintenance over such a period is not available for growth at the start of the period. This contrast in survival periods in B and G deficiency is probably related to the fact that the tissues of rats are not as easily depleted of vitamin G as of vitamin B (Graham and Griffith, '32). Vitamin G is present in the tissues of rats showing complete growth failure on a G-free diet (unpublished data). It may be that this vitamin is a tissue constituent which cannot be removed or these rats may have an unrecognized source of G which is adequate for maintenance but not for growth. The following report represents the first part of an investigation of the occurrence and function of vitamin G in the animal body and deals with the possibility of the absorption and utilization in the rat of vitamin G which may have been synthesized by microorganisms in the cecum.

<sup>1</sup> We wish to thank Mr. M. Sheppeck and Mr. E. E. Nixon for their assistance in this investigation.

The synthesis of vitamins B and G by the intestinal flora has been demonstrated and the necessity of preventing coprophagy has been recognized by numerous investigators. Recently, Guerrant, Dutcher and Tomey ('35) showed that the effects of coprophagy were much more marked if the carbohydrate of the diet consisted of dextrinized cornstarch rather than raw cornstarch, sucrose, lactose or glucose. An earlier paper (Bechdel, Honeywell, Dutcher and Knutsen, '28) demonstrated that appreciable amounts of vitamin B are synthesized by bacteria in the rumen of the cow and are absorbed from some portion of the alimentary canal. The early appearance of fatal deficiency signs on a B-free diet demonstrates that such a source of B is not available in the rat. It seemed that the question of an intestinal source of G in the absence of coprophagy might be answered by a study of the G requirement of young rats in which cecectomy had been performed. The cecum of the rat is relatively large and would appear to be a suitable incubator for vitamin-synthesizing organisms. With this in mind over 200 rats were cecectomized and their growth studied on various diets. After the completion of this work the paper of Guerrant, Dutcher and Tomey ('35) appeared in which a report was made of the effect of cecectomy in two rats. Their data indicated that the vitamin potency of the feces of an operated animal had been greatly reduced. In our experiments cecectomy was performed for a different purpose; i.e., to determine whether the rat regularly received significant amounts of vitamin G from the intact cecum.

#### EXPERIMENTAL

Young male albino rats were transferred to the experimental diet after attaining a weight of 49 to 51 gm. Such rats averaged 26 days of age. They were kept in single raised cages to prevent coprophagy. A record was made of the food consumption and of the rate of growth.

If cecectomy was performed the rats were operated 1 day after weaning. Recovery was rapid and these rats averaged 50 gm. in weight when 28 days of age.

The basal diet was the Evans and Burr mixture of purified casein, 25; sucrose, 75; and salt mixture, 4. In some experiments raw cornstarch replaced sucrose. Each rat received 0.2 cc. of fat daily, cod liver oil and lard being given on alternate days. Vitamin G was supplied in 0.4 gm. of autoclaved hog liver. Fuller's earth adsorbates, prepared from alcoholic extracts of rice bran or of wheat embryo, served as the vitamin B supplement. The combination of the vitamin B and G supplements permitted normal growth, thus demonstrating the presence of all of the necessary water soluble growth factors. In certain cases dried brewer's yeast was used as a source of the vitamins of the B complex.

#### THE EFFECT OF CECECTOMY ON GROWTH ON AN ADEQUATE DIET

The cecum is a dispensable organ as far as growth on the stock diet for a 100-day period is concerned. The average weight of twenty-four stock control male rats at the end of the 100-day experimental period was 284 gm. The average weight of thirty-nine cecectomized rats for the same period was 273 gm. The operated rats appeared normal in every respect and there was no evident physical difference in the feces of the two groups.

Twelve additional rats were cecectomized and fed the sucrose basal diet supplemented with fat and 0.5 gm. of dried brewer's yeast daily. This group averaged 211 gm. in weight after 50 days, an average daily increment of 3.2 gm. The absence of scurvy in this group was interesting because it was evident that freedom from scurvy in rats was independent of the cecum. The bulk of evidence at present favors the conclusion that rats synthesize vitamin C (Parsons and Hutton, '24). We are not aware of any experiments which exclude the possibility that rats may be supplied with this vitamin from intestinal microorganisms. The results with the above group rule out the cecum as a possible source of C and strengthen the belief that C is a metabolic product in the rat.

## THE EFFECT OF CECECTOMY IN COMPLETE B DEFICIENCY

Fatal polyneuritis was produced by feeding the basal diet and the daily supplement of 0.2 cc. of fat and 0.4 gm. of autoclaved liver. The average period before polyneuritis appeared in forty-four unoperated rats on the sucrose basal diet was 42 days. The period was shorter on the starch basal diet. In a group of fourteen cecectomized rats on the latter diet, three died before polyneuritic symptoms were observed, deaths occurring on the twenty-third, twenty-eighth and twenty-ninth days. The remaining eleven showed the usual signs after 16 to 38 days, with an average period of 29 days. In a control group of fourteen unoperated rats on the same diet, two died before signs appeared, on the twenty-seventh and twenty-eighth days, respectively. The remaining twelve developed polyneuritis within 25 to 42 days, with an average period of 35 days. Although polyneuritis and death occurred a few days earlier in the case of the operated rats, it is not believed that the difference between the two groups indicated any appreciable absorption of vitamin B from the intact ceca of the unoperated rats.

## THE EFFECT OF CECECTOMY IN COMPLETE G DEFICIENCY

G deficiency was produced by feeding the basal diet and the daily supplement of 0.2 cc. of fat and the fuller's earth adsorbate from either wheat germ or rice bran extract. The daily supplement of these adsorbates was adequate for good growth if autoclaved liver was also fed. The average weights of twelve cecectomized and thirteen unoperated rats after 70 days on the G-deficient sucrose diet were 50 gm. and 52 gm., respectively. The average weights of fourteen cecectomized and fourteen unoperated rats after 60 days on the G-deficient starch diet were 53 gm. and 46 gm., respectively. These results showed definitely that survival on G-free diets was not related to a cecal supply of vitamin G, since the cecectomized rats were in no way inferior to the unoperated controls.

Complete growth failure on a diet containing adequate supplements of vitamins A, B and D has been considered evidence

of the absence of G from the food mixture since the addition of autoclaved liver permitted normal growth. The signs of G-deficiency in young rats have been variously characterized by different investigators. Many believe that multiple factors are involved, particularly with respect to the types of dermatitis or pellagra-like symptoms which appear (Chick, Copping and Edgar, '35; György, '35). In our laboratory these symptoms have been observed to depend in part upon the carbohydrate of the diet. Young rats on the starch basal diet have never developed the brownish-red staining of the fur which is characteristic of those on the sucrose basal diet, thus confirming the work of Hogan and Richardson ('34). Evidently starch supplies a vitamin or some other dietary essential which prevents hemorrhage and the resulting staining of the fur. It may be possible that vitamin G, alone, is involved and that the amount required for growth is considerably more than that required for 'maintenance.' It appears doubtful, however, that vitamin G could be responsible for such a difference in the appearance of the two groups of rats and yet not affect the growth or survival of the groups. It is believed, therefore, that the picture of G deficiency in rats on the starch basal diet is more accurate than that observed on the sucrose basal diet. This picture is characterized by complete growth failure within 10 days; by inflammation and watering of the eyes within 10 to 20 days, followed by a sticking together of the eyelids; and, by survival for 50 days or more. Soreness of the skin on the face, around the ears, or inside the forepaws has rarely occurred. Of sixty-four rats surviving after 50 days on the G-deficient diet and showing complete growth failure only thirteen developed partial baldness. The eyes of twenty-six of these rats were examined at autopsy for evidence of cataract. Opacity of the lens was found in only two of these animals (Day, Langston and O'Brien, '31).

#### THE EFFECT OF CECECTOMY IN RATS ON DIETS LOW IN VITAMIN G

Although significant differences were not observed between the cecectomized and control rats in either B or G avitaminosis, it was thought that the effect of cecectomy might be

evident in more nearly normal animals, i.e., those receiving some B and G. For this purpose the sucrose basal diet was supplemented with cod liver oil, an adequate fuller's earth adsorbate of vitamin B and 50 mg. of autoclaved liver. The liver supplement prevented signs of G deficiency but permitted only subnormal growth. After 60 days, eleven cecectomized and twelve unoperated rats gained an average of 53 gm. and 58 gm., respectively.

In a second series both the sucrose basal and the starch basal diets were used and some of the rats were kept in cages

TABLE 1

*The effect of cecectomy on the rate of growth of young male rats during a 60-day period on a low B and G diet<sup>1</sup>*

SERIES	NUMBER OF RATS	AVERAGE GAIN IN WEIGHT	AVERAGE FOOD INTAKE <sup>2</sup>	REMARKS
		gm.	gm.	
190	14	38	263	Unoperated, sucrose basal, cages raised
200	14	34	251	Ceectomized, sucrose basal, cages raised
210	14	59	315	Unoperated, sucrose basal, cages on shavings
230	14	56	289	Ceectomized, sucrose basal, cages on shavings
500	28	36	271	Unoperated, starch basal, cages raised
510	29	29	253	Ceectomized, starch basal, cages raised
490	14	58	307	Unoperated, starch basal, cages on shavings
290	14	49	299	Ceectomized, starch basal, cages on shavings

<sup>1</sup> Basal diet supplemented with 100 mg. dried brewer's yeast daily.

<sup>2</sup> Total food consumed exclusive of fat supplement.

resting on shavings so that coprophagy was possible. Vitamins B and G were supplied in 100 mg. of dried brewer's yeast daily. With this supplement neither B nor G deficiency symptoms appeared but growth was subnormal. Vitamin G was the limiting factor in this yeast. The results of these experiments are shown in table 1. In every case the average gain in weight of the cecectomized rats was less than that of the unoperated controls. The differences, however, were hardly great enough to permit the conclusion that vitamin G was available in significant amounts from the intact cecum. In these experiments growth on the cornstarch basal diet was approximately the same as that on the sucrose basal diet. It

is noteworthy that coprophagy was of benefit to the operated as well as to the control rats. Vitamins of the B complex were present, therefore, in the intestinal contents of the cecectomized animals. It was evident, however, that this additional vitamin supplement was not available except through coprophagy.

#### DISCUSSION

The optimum requirement of vitamins B and G has been shown to be related to the composition of the basal diet by numerous investigators. These associated factors have been enumerated and discussed recently by Guerrant and Dutcher ('34) who have suggested that the nature of the food mixture may affect the vitamin requirement by influencing the microorganisms of the digestive tract. The experiments reported in this paper were performed in the hope that cecectomy might largely remove any effect of the intestinal flora and permit a more accurate determination of the metabolic need for vitamin G particularly. The experiments have failed to demonstrate a significant difference in the development of symptoms of either B or G deficiency in cecectomized and unoperated control rats. The synthesis of B or G by cecal microorganisms does not result in utilization of these substances by the rat if coprophagy is prevented. The long survival of rats on G-deficient diets and the difficulty in depleting the tissues of these rats of vitamin G are apparently not related to the presence of an intact cecum.

It is possible that there is utilization of vitamins produced by the flora of other portions of the intestinal tract. Indeed these experiments demonstrated that coprophagy benefited the operated rats. To what extent the large intestine compensated for the cecectomy cannot be stated definitely. There was some evidence that the colon undergoes change after the removal of the cecum. The intestinal tracts of operated animals were studied by the department of pathology for which we are indebted to Dr. W. D. Collier. Briefly, it was found that following cecectomy there was a marked dilatation of the first portion of the colon with a thinning and outpouching of



the wall. The fecal contents were fluid and of the consistency of the usual cecal material. The semi-solid fecal masses were formed farther down in the distal portion of the colon than is commonly the case.

#### CONCLUSIONS

1. Young rats grow normally for at least 100 days after removal of the cecum.

2. If vitamins B, C or G are synthesized by cecal micro-organisms, they are not utilized by the rat except by coprophagy.

3. The prolonged survival of young rats on G-deficient diets is not due to a cecal supply of vitamin G.

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## STUDIES ON GROWTH

### IV. THE VITAMIN B AND G CONTENT OF THE BODY TISSUES OF NORMAL AND EXPERIMENTAL RATS

WENDELL H. GRIFFITH

*Department of Biological Chemistry, St. Louis University School of Medicine,  
St. Louis*

#### THREE FIGURES

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In earlier experiments Graham and Griffith ('32)<sup>1</sup> found that adult rats were readily depleted of most of the vitamin B present in the tissues whereas a corresponding loss of vitamin G did not occur. Similar experiments have been performed with young rats in order to determine to what extent vitamin G can be removed from tissues during the prolonged survival period characteristic of rats on G-deficient diets. For this purpose the tissues of rats showing complete growth failure and other signs of G avitaminosis were fed as the vitamin G supplement to rats maintained on a diet lacking in G. For comparison, such tissues were also used as a vitamin B supplement. In addition, the vitamin B and G supplementing effect of the tissues of normal and of polyneuritic rats were investigated.

#### EXPERIMENTAL

Young male albino rats, 49 to 51 gm. in weight and averaging 26 days of age, were used. The rats were kept in individual raised cages with bottoms of wire mesh to prevent coprophagy. The experimental diet was the Evans and Burr

<sup>1</sup> In this paper, line 24 on page 696 should read "more rapidly on diets low in B<sub>1</sub> than on diets low in B<sub>2</sub>."

mixture of purified casein, 25; sucrose, 75; and salt mixture, 4. Two-tenths cubic centimeter of fat was fed daily, cod liver oil and lard being used on alternate days.

Rat tissues which were used as supplements were prepared in the following way. The animals were decapitated and skinned. The paws, stomach and intestines were discarded. The carcasses were then hashed in a grinder, dried in a warm air dryer at 50°C. and pulverized. In this manner, dried tissues were obtained from the carcasses of seven groups of rats.

Tissue I (6.0 gm. of dried residue per rat) represented normal rat tissue and was prepared from 122 rats averaging 50 gm. in weight. These rats were similar to those used in the feeding experiments.

Tissue II (6.8 gm. of dried residue per rat) represented the tissues of rats with B deficiency. Forty-three rats were fed the basal mixture supplemented with 0.4 gm. of autoclaved hog liver daily. This supplement was adequate for normal growth if vitamin B was also present. The rats were killed when polyneuritic symptoms appeared. The average interval before the paralysis became evident was 45 days and the average weight of the polyneuritic rats was 45 gm.

Tissue III (7.0 gm. of dried residue per rat) represented the tissues of rats with G deficiency. Twenty-seven rats were fed the basal mixture supplemented with 75 mg. of a fuller's earth adsorbate of an alcoholic extract of wheat germ. This vitamin B supplement was adequate for normal growth if autoclaved liver was used as a source of vitamin G. These rats were killed after 40 days and averaged 47 gm. in weight at the end of the experimental period.

Tissue IV (5.4 gm. of dried residue per rat) was the same as tissue III except that the rats were given only 18 mg. instead of 75 mg. of the fuller's earth preparation of vitamin B. This was done in order to minimize the possible effect of any traces of G in the activated earth. These rats, therefore, received a supplement very low in B and lacking in G. The twenty-seven rats were killed after 40 days and averaged 42 gm. in weight at the end of the experimental period.

Tissues V (7.8 gm. of dried residue per rat) and VI (6.4 gm. of dried residue per rat) were also similar to tissue III. Twenty-eight rats were fed the basal mixture supplemented with the activated fuller's earth. Fourteen of these rats were killed after 25 days and averaged 50 gm. in weight (tissue V). The remaining fourteen were killed after 46 days and averaged 45 gm. in weight (tissue VI).

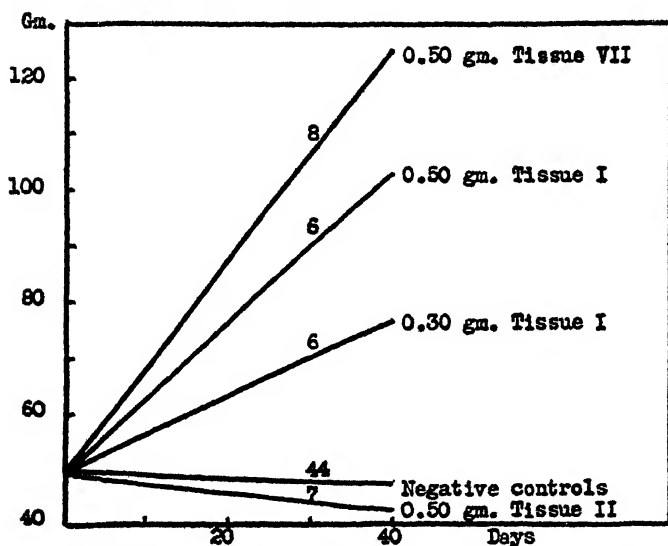


Fig. 1 The average increase in weight in a 40-day period of rats on a B-free ration supplemented with dried rat tissue as a source of vitamin B. The figure on each line shows the number of rats in the group.

Tissue VII (8.9 gm. dried residue per rat) represented the tissues of rats which had survived 100 days on the basal mixture supplemented with the fuller's earth preparation of vitamin B. The forty-five rats averaged 58 gm. in weight and showed varying degrees of hair loss. The eyes of thirty-two of these animals were examined for cataract with negative results.

The dried tissue was mixed with small amounts of the basal diet and measured portions were fed daily. No difficulty was experienced through refusal of rats to eat such supplements.

The vitamin B potency was determined by feeding the tissue preparation to rats receiving the basal mixture, fat and vitamin G in the form of autoclaved hog liver. The vitamin G potency was determined by feeding the tissue to rats receiving the basal mixture, fat and vitamin B. The latter supplement was a previously tested activated fuller's earth adsorbate of an 80 per cent alcoholic extract of wheat germ.

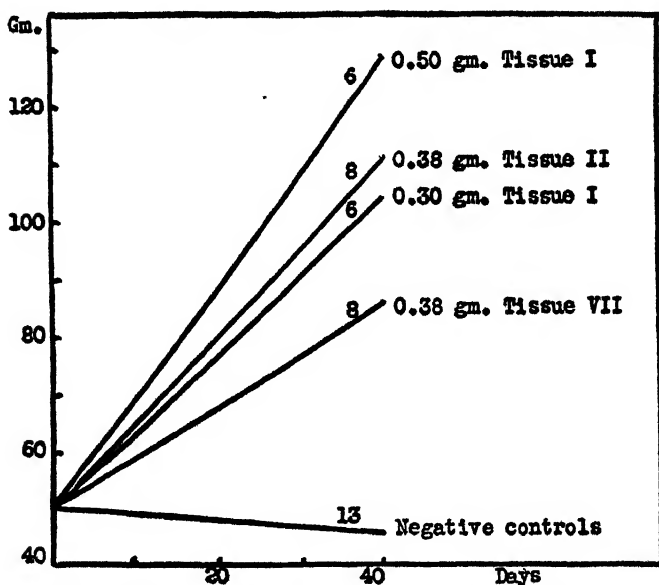


Fig. 2 The average increase in weight in a 40-day period of rats on a G-free ration supplemented with dried rat tissue as a source of vitamin G. The figure on each line shows the number of rats in the group.

The quantities of normal tissue (tissue I) which permitted definite growth as B and G supplements were determined first. Similar amounts of the experimental tissues were then used. No attempt was made to compare accurately the vitamin potency of the experimental tissues with that of the normal tissue because the weight of dried residue per rat differed with each of the seven preparations. These differences were due, probably, to the varying proportions of skeletal tissue to soft vitamin-containing tissue in the rats at the end of the

experimental periods. Therefore, the weight equivalents which were used as supplements actually represented unequal quantities of the soft tissues such as muscle or liver tissue. The amount of dried tissue required as a daily supplement for normal growth was not found because of the considerable quantity of material necessary for such determinations.

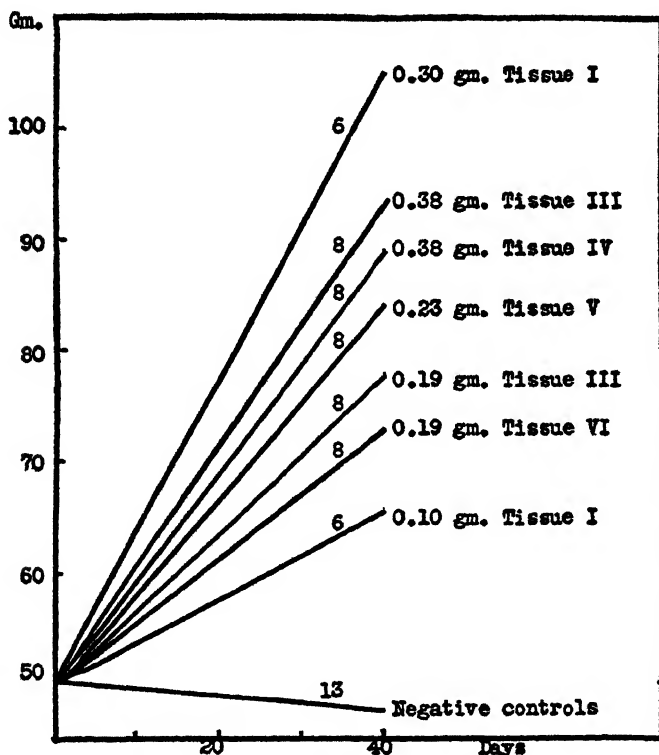


Fig. 3 The average increase in weight in a 40-day period of rats on a G-free ration supplemented with dried rat tissue as a source of vitamin G. The figure on each line shows the number of rats in the group.

Figure 1 shows the effect of feeding tissues I, II and VII as the vitamin B supplement. It is evident that vitamin B was present in the tissues of normal rats (tissue I) and of rats maintained on a food mixture lacking in G but containing B (tissue VII). However, the tissues of polyneuritic rats

(tissue II) were completely deficient in vitamin B since the rats fed tissue II as a vitamin B supplement failed to grow and developed polyneuritis as did the negative controls.

Figure 2 shows the growth which resulted when these same tissues were used as the vitamin G supplement. As was found in our earlier experiments the tissues of normal rats (tissue I) were richer in G than in B. Three-tenths gram of this tissue as a source of G permitted the same rate of growth as 0.5 gm. as the source of B. The tissues of polyneuritic rats (tissue II) which were devoid of any effect as a B supplement were potent as a G supplement. The tissues of rats maintained on a G deficient diet for 100 days (tissue VII) still contained vitamin G although the quantity was apparently less than that in normal tissues.

Tissues III to VI (fig. 3) were prepared from rats with varying degrees of G avitaminosis. Tissues III and IV had about the same potency although the former was obtained from rats maintained on a diet adequate in B and lacking in G and the latter from rats receiving a supplement low in B and lacking in G. Tissues V and VI were obtained from rats fed the G-deficient diet for 25 and 46 days, respectively. All of these tissues contained vitamin G.

#### DISCUSSION

These results with the tissues of young rats were in agreement with those previously reported for the tissues of adult rats (Graham and Griffith, '32). The prolonged survival of rats on diets lacking in vitamin G appears, then, to be related to the retention of G in the body tissues, whereas no such retention occurs in the case of vitamin B. Experiments are in progress to determine to what extent vitamin G may be stored as excess G and whether other tissues than the liver are involved.

The difficulty in depleting tissues of vitamin G might be explained by the presence of a small amount of this vitamin in the food or by the utilization of G synthesized by micro-organisms in the alimentary tract and absorbed therefrom

or by efficient use of the original vitamin of the tissues so that minimum waste or loss occurs. The first explanation was not considered probable because some growth would have resulted in rats which were fed the G-deficient diet if the food supplied the G actually found in the tissues of these rats. Growth failure always occurred on the G-deficient ration. The second explanation likewise appeared improbable because it has been shown that the cecum, at least, does not serve as a source of vitamin G (Griffith, '35). The third explanation appeared more plausible and it is suggested that vitamin G may be a tissue constituent which cannot be readily removed. If it is true that vitamin G is a component of certain body tissues, growth would require increasing amounts of the vitamin in the food. Booher, Blodgett and Page ('34) have observed that there is a close correlation between the requirements for vitamin G and the body weights of growing animals. The decrease in food consumption and resulting loss of weight occurring in adult rats which have been placed on a G-deficient diet indicates that vitamin G may be necessary as an appetite stimulant as well as a tissue constituent. The influence of G on the consumption and utilization of food has previously been emphasized (Graham and Griffith, '33).

#### CONCLUSIONS

1. Young rats maintained on a B deficient ration were readily depleted of most of the vitamin B originally present in the tissues.
2. A corresponding loss of vitamin G did not occur in young rats on a G-deficient ration.
3. The prolonged survival of rats on a G-deficient ration is related to the retention of tissue vitamin G.
4. It is suggested that vitamin G may function in part as a tissue constituent.



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## VARIABILITY OF VITAMIN D RESPONSE WITH TEMPERATURE OF ENVIRONMENT <sup>1</sup>

D. TOURTELLOTTE AND W. E. BACON

*General Foods Corporation, Battle Creek, Michigan*

TWO FIGURES

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Anyone who has followed the vitamin D assay closely for any extended period of time has certainly encountered all manner of variation, some of which can be significantly related to definite factors and others which remain obscure. Probably the most exhaustive study of the vitamin D determination has been reported by a group of workers from the Lister Institute and the National Institute for Medical Research, London, Hume et al. ('32), Bourdillon et al. ('32). They have been extremely careful in their studies of the preparations which have served as the International vitamin D standards. In this connection they have reported the errors involved in the different vitamin D assay procedures.

In addition to the errors inherent in the methods used they also found a variation in the sensitivity of the stock of rats to rachitogenesis from time to time which could not be related to the vitamin D. Quoting from Hume ('32):

Whether such a variation in the response of the experimental animals at different times is due to variation in the animals' reserves of vitamin D or to variation in the rickets-producing power of any of the constituents of the basal diet or to some other unrecognized cause, is at present not known, but it is one of the greatest causes of difficulty in the biological estimation of vitamin D.

<sup>1</sup> Presented before the American Institute of Nutrition, April 10, 1935, at Detroit.

In a test of the stability of the International vitamin D preparation extending over 21 months, Bourdillon encountered maximum fluctuations in excess of 300 per cent in the apparent potency. From their results, however, they concluded that there was no appreciable deterioration in vitamin D potency and that the major variations were due to changes in the general sensitivity of the stock.

Bills and co-workers ('31) have reported a critical study of the line test results accumulated from their experience with routine laboratory assays of vitamin D-containing materials. Bills has treated the errors within any assay group but does not mention a variation in sensitivity of the test animals from time to time. In a private communication Doctor Bills has told us that he has not encountered this difficulty under his present conditions for conducting vitamin D assays.

We have standardized so far as possible in the conduct of our animal colony, for ruling out the variations due to dietary differences. We purchase a year's supply of grains and have established a constant source of supply for other constituents in the rachitogenic diet. The rats used in our colony are the McCollum inbred strain.

We have attempted to take care of the matter of environmental temperature by placing a thermostatic control on our heating system. However, this does not take care of the heat drawn from other parts of the building by our ventilating fan nor does it take care of excessive summer temperatures. We have no means of regulating humidity although it must certainly be a factor in the control of laboratory animal quarters. In this connection several laboratories in this country have been equipped for year round control of temperature and humidity.

We use the McCollum line test procedure as modified by Bills. The 5-day assay procedure as introduced by Bills possesses the advantage of speed and reliability over other existing vitamin D methods. We use the McCollum rachitogenic diet no. 3143. The autopsy and line test procedure are conducted by the usual technic. The tibia healing is graded by

direct observation of the stained bone according to the scale devised by Bills. Since the four grades of healing bear no simple numerical relation to each other he has developed a graphical relationship of healing to potency which is another feature in the assay technic and which gives an exact potency factor for any degree of healing within the sensitive range of the test.

We have used the 5-day assay procedure since the latter part of 1931. Since 1933 and until the U.S.P. vitamin D standard became available we used cod liver oil as a control standard for every vitamin D assay which we made. Our auxiliary standard was checked against the U.S.P. vitamin D standard when it became available and fortunately they agreed very closely. Thus since early in 1933 until the present date we have controlled all vitamin D assays with either the auxiliary standard or with the U.S.P. standard containing 95 U.S.P. units per gram.

Although we have been aware of a fluctuation in the sensitivity of our test animals prior to the use of standardized preparations as control for our assays we had no means of knowing its magnitude. The accumulation of results from the assay of reference standards showed an apparent fluctuation in potency of approximately 400 per cent. Our first notion was that the fluctuation was in some way a seasonal problem since the results showed their greatest depression during the summer months. Fortunately we had kept daily 24-hour records of the laboratory temperature and when our potency values were correlated with these we found that the greatest depressions in apparent potency coincided with high laboratory temperatures during the rachitogenic and test periods (fig. 1). A correlation diagram has been constructed (fig. 2) relating the apparent potency with the mean daily temperature of the combined rachitogenic and test periods (26 days) and it shows that laboratory temperatures of 80°F. and above give rise to considerable variation in the apparent potency of standard reference materials.



To show that the fluctuation during periods of high laboratory temperature is due to an increased severity of rachitogenesis we have been able to conduct satisfactory vitamin D assays on rachitic animals after only 11 to 13 days on the rickets-producing diet, as against the regular rachitogenic period of 21 days. Examination of these 11- to 13-day

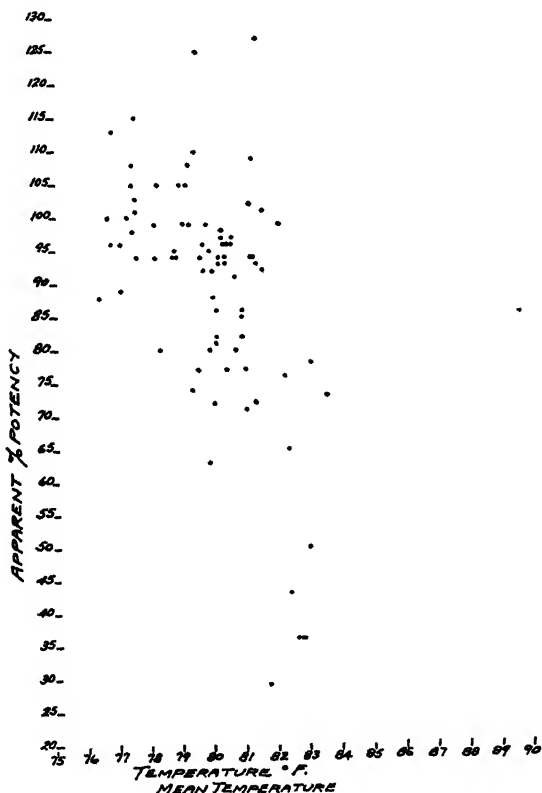


Fig. 2 Correlation diagram, relating mean temperature of rachitogenic and test periods with apparent assay potency of reference vitamin D standard.

rachitic animals showed a satisfactory grade of line test rickets.

We have also been able to reduce the severity of rickets produced in the regular rachitogenic period by reducing the Ca:P ratio of the diet. The McCollum diet no. 3143 regularly contains 3 per cent  $\text{CaCO}_3$  and a Ca:P ratio of about

5:1. By reducing the  $\text{CaCO}_3$  to 2 per cent good line test rickets have been produced in 21 days during these periods of uncontrollably high temperatures.

In conclusion, we have found that to a certain extent the variation in the sensitivity of the rachitic test animals is due to fluctuating laboratory temperatures during the rachitogenic and test periods. An increased severity of rachitogenesis results in vitamin D test animals when they are subjected to laboratory temperatures of  $80^\circ\text{F}$ . or above. We believe that it is fully as desirable to define a standard rachitic test animal in terms of response to a definite amount of a standard vitamin D preparation as it is to prescribe the exact conditions for rachitogenesis and methods for administering the test preparations.

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## EDITORIAL REVIEW

# THE ABSORPTION AND UTILIZATION OF CARBOHYDRATES

H. B. PIERCE

*Department of Vital Economics, University of Rochester, Rochester, N. Y.*

Carbohydrates have three primary functions in nutrition, 1) to furnish energy and heat, 2) to build glycogen as a reserve store for energy and 3) to make fat. In addition, carbohydrates are used to fulfill many other minor needs of the body. They function in the synthesis of certain cerebro-sides and glyco-proteins and in stimulating the production and flow of insulin, intestinal juice and possibly bile. They protect the liver against damage resulting from chloroform poisoning. Evidence is available showing that certain carbohydrates cause marked changes in the intestinal flora. The sparing action of carbohydrate on protein metabolism is well recognized, and in bacterial culture media, the addition of sugars tends to prevent or limit the formation of putrefactive products. Another role is their entrance into certain phases of mineral metabolism.

As this subject is one of tremendous scope, the present review will be limited as far-as possible to considerations involving normal organisms and the more common carbohydrates. Even with these limitations, the great volume of literature necessitates the omission of many references.

## ABSORPTION OF CARBOHYDRATES FROM THE ALIMENTARY TRACT

The absorption of carbohydrates from the gastro-intestinal tract of man and animal has been of interest to many investigators. In this section, their absorption from the stomach and



small intestine will be reviewed. The experimental procedures used are not at all similar, a fact which undoubtedly accounts for the variable results reported in the literature.

*Absorption from the stomach.* Data have been obtained, a) by use of animals with gastric fistulas, b) by tying the stomach at the pylorus and recovering the sugar introduced at a later hour, c) by determining the sugar content of blood flowing from the stomach, and d) by measuring differential changes in the concentration of a sugar and non-absorbable substance mixed together and recovered after the period of absorption. We have found the last method unsatisfactory in determining sugar absorption from the intestine and Maddock, Trimble and Carey ('33) have commented on the complexity of the computations following employment of this technic.

A review of the literature concerning the gastric absorption of sugars having been presented recently by Maddock, Trimble and Carey ('33) only brief mention of results will be presented here. It is the contention of some workers that glucose is absorbed from the stomach, particularly if in alcoholic solution, but others have proved that no glucose or sucrose is absorbed under these circumstances. Roth and Strauss (1889) in discussing their results (p. 178) stated *Der Magen ist also ein Organ der Sekretion, welches zur Resorption ungeeignet ist.*"

Experiments performed by Macleod, Magee and Purves ('30) on urethanized rats led them to conclude that little or no glucose was absorbed from stomachs ligated at the pylorus. Maddock, Trimble and Carey ('33) reached the same conclusion as they found no increase in the sugar content of blood in gastric veins of dogs after placing glucose in their stomachs. An average of 99 per cent of the glucose introduced was recovered after absorption periods of 1 to 2 hours.

The widely accepted view of today is that the amount of sugar absorbed from the stomach under conditions of normal digestion is small and perhaps insignificant.

*Absorption from the small intestine.* Although some of the procedures outlined under 'absorption of the stomach' have

been used in studying intestinal absorption of carbohydrates, the majority of the experiments have been carried out by one of three methods, a) by use of isolated segments in anesthetized animals or Thiry or modified Thiry loops in unanesthetized animals, b) by use of either all or the greater part of the gastro-intestinal tract in anesthetized or unanesthetized animals and c) by determining the sugar content of the blood during absorption. Here again differences in technic have made it difficult to compare and correlate data from various laboratories. Anesthesia, the section of the intestine used, the condition and age of the animal, and the effect of the stomach or its secretions on the sugar solution have not been taken into consideration in the evaluation of many data. Although Cori ('31) stated that the blood sugar curve was not a measure of the rate of absorption, Magee and Reid ('31) found the percentage increases in blood sugar curves were in accord with absorption coefficients (see p. 692, this text) obtained in both anesthetized and unanesthetized animals. While this conclusion may apply to their particular experiments, it has no standing as a general proposition, as is shown further on in this paper.

For the sake of clarity, the subject of intestinal absorption of carbohydrates will be divided into five parts; 1) absorption rates of different sugars, 2) uniformity of absorption rates over periods of time, 3) effect of concentration of sugar solutions on absorption rates, 4) effect of the section of the tract used on absorption rates and 5) effect of the stomach and its secretions on absorption rates.

*1. Absorption rates of different sugars.* It seems natural to assume that there is a relationship between the rates of absorption and utilization of carbohydrates. Early results were obtained by use of isolated segments of various sections of the intestine of anesthetized or unanesthetized animals.

From data presented in papers by Nagano ('02 a and b), Röhmman and Nagano ('03), Cori ('25), Verzár ('35) and Feyder and Pierce ('35) it may be concluded that the disaccharides are absorbed more slowly from the intestine than are

the monosaccharides, with the exception of fructose. Glucose and galactose are absorbed at approximately the same rate which is considerably higher than that for fructose, while the disaccharides sucrose and maltose are removed more rapidly than lactose. In solutions containing both glucose and galactose, glucose passes through the intestinal wall more rapidly than galactose.

2. *Uniformity of absorption rates over periods of time.* Hédon ('00) determined the rate of absorption of glucose for 0.5 to 6.0 hours and his results revealed an initial rate of 1.25 gm. per hour while 6 hours later this had dropped to 0.75 gm. per hour.

A number of years later Cori ('25) fed rats known quantities of hexoses and pentoses by stomach tube and after definite intervals of time sacrificed the animals, recovering the unabsorbed sugar from the gastro-intestinal tract. As a measure of the amount of sugar absorbed he used a 'coefficient of absorption' defined as that amount of sugar absorbed per 100 gm. body weight per hour. The coefficient remained constant from hour to hour or until all of the sugar had disappeared from the tract. After prolonged fasting the absorption rate remained constant, but at a lower level (Cori, '31). The agreement of these coefficients among groups was less variable in Cori's data than in those of Pierce, et al. ('29), Feyder and Pierce ('35), MacKay and Bergman ('33 a and b) and Magee and Reid ('31). In our data all results were included in the average provided no abnormalities were observed during the absorption period. The greatest range for absorption coefficients was noted in results for the 1-hour period. For the sake of comparison of various coefficients of absorption of glucose (milligrams per 100 gm. body weight per hour), the following table is inserted:

	<i>Hours</i>		
	1	2	3
Cori ('25)	196	161	178
Cori, et al. ('29)	203	235	220
Pierce, et al. ('29)	236	229	168
Feyder and Pierce ('35)	213	181	160

Magee ('30) stated that Auchinachie, Macleod and Magee in unpublished work had, in general, confirmed Cori's results for sugar. Holtz and Schreiber ('30) also reported that the absorption of glucose took place at a constant rate. Trimble and Maddock ('34) introduced glucose solutions directly into the lumen of the duodenum of dogs and concluded that the sugar was absorbed at a uniform rate over a period of hours.

Although the findings of Cori ('25) have been confirmed by several workers just cited, others have obtained results which are not in agreement with his. Pierce, Osgood and Polansky ('29) investigating the rate of absorption of glucose from the alimentary tract of rats with the Cori ('25) technic, found there was a falling off in the absorption rate during the second and especially the third hour after feeding. Cori, Cori and Goltz ('29) pointed out that Pierce, et al., had not fed enough sugar to permit the sustenance of a uniform rate of absorption for a 3-hour period. This seemed a logical criticism in light of the data submitted. Cori et al. ('29) on the basis of new data submitted, reaffirmed the former conclusion that glucose was absorbed at a constant rate. Magee ('30) stated, "Cori's opinion may then be taken as substantially correct; but all the published results including his own, show a slight falling off in the rate as absorption proceeds."

Data submitted by Burget, Moore and Lloyd ('32, '33), Ravdin, Johnston and Morrison ('33 b), MacKay and Bergman ('33 a and b) and Feyder and Pierce ('35) failed to show a linear relationship when the amount of sugar absorbed was plotted against time. MacKay and Bergman ('33 a and b), Pierce, et al. ('29) and Feyder and Pierce ('35) noted that there was a closer agreement between the amount of glucose absorbed per unit of surface than per unit of weight, a finding disagreeing with that of Maddock, Trimble and Carey ('33). MacKay and Bergman ('33 b) in discussing the results of Trimble, Carey and Maddock ('33) suggested that the uniformity in the rate of absorption of glucose noted by the latter workers was due to the increasing amounts of glucose fed for the longer absorption periods. In replying, Maddock, Trimble and Carey ('33) selected data from their earlier paper indi-

cating that the dosage of glucose had nothing to do with the rate of its absorption.

3. *Effect of concentration on absorption rates.* Nagano ('02 a) and Omi ('09) using comparatively dilute solutions of several sugars, obtained results demonstrating that the total amount absorbed increased as the solution became more concentrated, although the percentage of the total absorbed decreased.

London and his associates ('08) approached the question of absorption in relation to concentration by an entirely different method. They introduced solutions of glucose ranging in concentration from 4.6 to 79.7 per cent into a fistula leading to the duodenum and later recovered the unabsorbed sugar from a fistula in the ileum. While a uniform absorption of approximately 0.4 gm. per kilo per hour prevailed from glucose solutions ranging from 11.5 to 37.5 per cent concentration, their total data showed absorption rates varying from approximately 0.2 to 0.9 gm. per kilo per hour. Although Cori ('25) concluded that the rate of absorption of hexoses was independent of the absolute amount and also of the concentration of the sugar present in the intestine, his own data (table III, p. 699) demonstrated that by increasing the amount of glucose fed per 100 gm. body weight from 0.819 to 1.459 gm., the coefficient of absorption rose from 143 to 184, a difference of 30 per cent.

In several different types of experiments, Auchinachie, Macleod and Magee ('30), Macleod, Magee and Purves ('30) and Magee and Reid ('31) obtained data showing that optimal absorption of glucose took place from 0.75 M (approximately 13 per cent) solutions of the sugar. Magee and Reid ('31) found that if the 0.75 M glucose was prepared in 0.2 per cent phosphate at pH 7.0, the absorption rate of the sugar increased by 50 per cent.

Glucose solutions of different concentrations are diluted to approximately the same percentage composition prior to their absorption from the intestine, according to Ravdin, et al. ('33 a). The solutions were administered by the Cori ('25) technic. Thus, they found that the glucose solution in

contact with the absorbing surface of the intestine had approximately the same concentration irrespective of that of the solution fed. It was believed by these authors that their failure to confirm Cori's ('25) results with respect to the effect of concentration upon absorption rate might have been due to neglect of dilution factors, introduced by the stomach, in that their solutions were introduced directly into the intestine.

Trimble, Carey and Maddock ('33) adapting Cori's technic to dogs, found no definite relationship between variations in concentration or absolute weights of glucose ingested and the rate of absorption. Trimble and Maddock ('34) reported that irrespective of the concentration of the glucose solutions used (3 to 32 per cent) the rates of absorption always averaged 0.92 gm. per kilo per hour for periods up to 3 hours, the sugar having been introduced directly into the lumen of the duodenum. Where the concentration of the glucose solution was low and the volumes of fluid introduced large, the Woodyatt type of pump was used. The use of this technic would require a consideration of possible effects of intra-intestinal pressure on absorption.

More recently, Verzář ('35) investigated the effect of concentration on the rate of absorption of several sugars from the intestine in urethanized rats. He selected a section of upper jejunum 30 cm. in length, washed it out with saline and after 1 hour introduced the sugar solution. One hour later, the animals were killed and the sugar remaining in the segment washed out and determined. Verzář concluded that the re-sorption of glucose and galactose from their solutions (1 to 14 per cent) was independent of their concentration, but this did not hold true for fructose, xylose, sorbose or mannose, where more sugar was absorbed with the more concentrated solutions. He believed that the absorption of glucose and galactose took place through active cell processes, while that of the other sugars studied resulted from pure diffusion.

4. *Effect of the section of intestine used on absorption rates.* It is generally conceded today that absorption from the upper part of the intestine is greater than from the lower. Röhmann and Nagano ('03) studying absorption rates of the disac-

charides from Vella fistulas of jejunum and ileum and later isolated loops of various sections of dog intestine, obtained data which revealed a more active absorption from jejunal than from ileal segments. The following values may be taken as illustrative of results obtained with 5 per cent solutions of disaccharides introduced into 30 cm. lengths and allowed to remain 1 hour.

	<i>Grams absorbed</i>	
	<i>Jejunum</i>	<i>Ileum</i>
Sucrose	2.39	0.95
Maltose	1.56	0.67
Lactose	0.75	0.26

Omi ('09) confirmed this point with 1 to 8 per cent sucrose solutions, always finding more absorption from the jejunum than from the ileum.

5. *Effect of the stomach and its secretions on absorption.* There can be no question that the emptying time of the stomach plays a role in affecting sugar absorption from the gut. Adam ('24) using infants, and Holtz and Schreiber ('30) using dogs, observed, by use of fluoroscopic methods, the movement of sugar solutions containing  $\text{BaSO}_4$  or colloidal  $\text{SiO}_2$  through the alimentary tract. Both saw the delayed passage of such solutions into the intestine, and the latter reported a closure of the pylorus for as long as 20 minutes in the midst of an absorption period. Macleod, Magee and Purves ('30) also Ravdin, Johnston and Morrison ('33 a) noticed that 1 to 2 hours after feeding glucose to animals, and while their stomachs still contained noteworthy quantities of sugar, little or none was found in the intestine. Macleod et al. ('30) also noted that the rate of discharge of sugar solutions through the pylorus varied inversely as their concentrations. As Cori ('31) pointed out, more work is required to determine whether the emptying time of the stomach is a limiting factor in absorption. Certainly the results cited above suggest strongly that pyloric activity must be considered in studies such as these.

#### ASSIMILATION OF CARBOHYDRATES

Many factors, such as endocrine disturbances, state of nutrition, age and sex of subject, fevers, pregnancy, fatigue,

activity, and temperature, may influence the assimilation of carbohydrates. Insofar as possible, the results of experiments on normal humans or animals will be reviewed and only brief summaries of some of the more important investigations can be presented.

As soon as sugar passes through the intestinal wall, it enters the blood stream and one quite naturally expects to find a rise in blood sugar levels. The rise and fall of blood sugar, during the earlier part of an absorption period at least, is dependent upon the relative rate of inflow of sugar from the intestine and the rate of its subsequent removal from the blood by the tissues. When large amounts of sugar are ingested, it is not surprising that the rate of absorption should exceed the rate at which the tissues can remove them. On the other hand, it is not clear why the blood sugar curve should fall to normal and below while the rate of absorption from the gut is still high. Apparently in the normal subject, some dormant factor awakens at a certain time after taking sugars and checks hyperglycemia.

Much of the earlier work in this field was unreliable due to the non-existence of satisfactory quantitative methods for the determination of blood sugar. Thus the assimilation limit of carbohydrates was determined by finding what amount of sugar could be fed without having any excreted in the urine, no attention being paid to blood sugar levels.

Macleod ('16) determined this 'assimilation limit' of glucose by feeding it to fasting subjects and testing the urine for sugar at frequent intervals. The majority of normal healthy individuals showed no glycosuria from a dose of 200 gm. With increasing doses up to 500 gm. more and more subjects had glycosuria. As much as 300 gm. invert sugar or sucrose has been fed to men with no evidence of urinary sugar. Field ('19) fed 100 gm. doses of various sugars to fasting adult Negroes, plotting curves for blood sugar and testing the urines for sugar at frequent intervals. With this amount of any of the sugars no glycosuria was exhibited. Glucose, maltose and mannite gave quite similar curves, the height being reached 1 hour after ingestion. The peak for the



mannite curve was not as high as for the two sugars. The rise was not so rapid with sucrose, the largest amount being present at the end of 2 hours. Lactose had practically no glycemic action. Mendel and Jones ('20) fed healthy rabbits 50 cc. of different sugar solutions varying in concentration, by stomach tube. After 3½ hours, the urine was tested for sugar and if no reducing substance was present, the amount of sugar for the next dose was increased. The smallest amount of carbohydrate which resulted in glycosuria was recorded as the 'assimilation limit' and these, in grams per kilo, were given as follows: sucrose 7.5, fructose 8.0, glucose 13.0, maltose 17.1, dextrin 16.1. These data were partially confirmed and made more complete by Ariyama and Takahasi ('29). Jones ('20) found that glucose raised blood sugar the most, dextri-maltose and dextrin following in the order given. The results with sucrose were somewhat erratic. Fructose, of the sugars used, was the only one which failed to raise blood sugar.

Folin and Berglund ('22) have reviewed additional papers dealing with the older work on sugar assimilation and have noted the many conflicting points of view expressed by numerous workers in the field. Jacobsen ('13) was one of the first to use an accurate method (Bang's micro method) in an attempt to correlate blood sugar levels with urinary sugar excretion. He used starch, glucose, protein, fat, and fat with starch in his experiments with normal and pathological subjects. Normal subjects were given either 100 gm. glucose or 167 gm. of white bread before or 2 to 3 hours after breakfast. At 15-minute intervals after the ingestion of the carbohydrates, sugar was determined in the blood and in urine samples, when obtainable. Eight of his fourteen subjects exhibited glycosuria after ingesting 100 gm. glucose, this commencing when blood sugar increased to 160-170 mg. per cent. The blood sugar level rose rapidly reaching a peak in from 15 to 45 minutes and returned to the original level some 2 to 3½ hours later. With 167 gm. white bread, seven of the fourteen subjects showed glycosuria, the blood-sugars reaching approximately the same level as noted with glucose, but returning to normal more slowly. Not being willing to accept

Jacobsen's figures as the standard for normals, Hamman and Hirschman ('17) pointed out that Hopkins ('15) after feeding 100 gm. glucose to normal persons, found the blood sugar to reach maximal levels of from 110 to 156 mg. per cent, but no tests were made on the urine. Hamman and Hirschman gave fasting subjects 100 gm. glucose in lemonade after a night's fast and noted that blood sugar rose rapidly to approximately 0.15 per cent, usually within 30 minutes, and returned to the original level within 1 to 2 hours. The renal threshold for glucose in normals was found to be 0.17 to 0.18 per cent. Folin and Berglund ('22) really explained the variable results of different workers when they said that the hyperglycemia just adequate for the production of glycosuria, if produced at all, lasted for so short a time that one seldom succeeded in collecting urine samples of that particular period.

Goto and Kuno ('21) published results of a series of threshold experiments on fifty-three Japanese subjects, giving them 100 gm. of glucose in 250 cc. of water after a night's fast. Thirty-three of the fifty-three subjects excreted sugar, the highest amount being 0.8 gm. The average blood sugar level of those persons not excreting glucose was 142 mg. per cent and of those excreting sugar 160 mg. per cent. With all subjects the alimentary hyperglycemia reached a maximum 40 to 60 minutes after the test and became normal within 3 hours.

The object of the experiments of Folin and Berglund ('22) was to determine the relationship between concentration of sugar in the blood and its elimination in the urine. They used glucose, fructose, galactose, lactose, dextrin and starch as sources of carbohydrate. When 200 gm. glucose were ingested by normal persons, blood sugar did not rise above the threshold and no glucosuria was noted. None of the other carbohydrates ingested raised blood sugar to the same extent as glucose, a point which other investigators have questioned and which will be discussed later. Although Folin and Berglund found that galactose and lactose had little hyperglycemic action, they noted a marked excretion of these sugars in the urine. Blood sugar fell even when absorption of sugar from the intestine was still going on. This was believed to be

due to the removal of sugar from the blood by the tissues rather than to glycogen formation. The subsequent hypoglycemia noted was a result of a decreased need for sugar transport from one tissue to another.

A comparison of the concentrations of sugar in arterial and venous blood during active absorption of several sugars from the intestine of humans was made by Foster ('23 a). The 'glycemic response,' following ingestion of different sugars, was noted by determining the sugar content of both venous and arterial blood, and was found to be dependent upon the carbohydrate used.

In commenting upon these and other results, Foster ('23 b) said that when a large amount of sugar was ingested, it was not surprising that the rate of absorption exceeded the rate at which tissues could remove it, but it was not clear why the curve returned to normal while the rate of absorption from the gut was undoubtedly still high. A further oddity was the continued fall of blood sugar even below the original level after glucose, but not with fructose or galactose. Further, it was not clear why a second dose of glucose failed to cause the same rise in blood sugar as noted with the first. Foster believed that the ingestion of glucose and the subsequent hyperglycemia stimulated insulin production which in turn accelerated both glycogen synthesis and oxidation of sugar, this being an idea supported in whole or part by the findings of Bornstein and Holm ('22). Soskin and Allweiss ('34) and Soskin ('35) do not believe the stimulation of insulin production is the correct explanation of the hypoglycemia, but detailed results are not yet available for review. Foster suggested that the carbohydrate oxidized and from which glycogen was formed, was not ordinary  $\alpha$  or  $\beta$  glucose but some substance formed when ordinary glucose was acted upon by insulin. This substance, he thought, was more readily formed from fructose, perhaps without the aid of the pancreatic hormone. As is seen, Foster's conclusions are quite different from those of Folin and Berglund ('22).

Several workers have observed that of the three common hexoses galactose produces the most marked hyperglycemia

followed by glucose and fructose. Glucose, but not fructose, given in equal quantity with galactose, reduced the hyperglycemia of galactose to a level slightly greater than that of glucose when given alone. This effect of glucose was noted by Folin and Berglund ('22) and later by Reinhold and Karr ('27).

It was found by Reinhold and Karr ('27) that galactose, maltose, sucrose, starch, lactose and fructose were effective in the order named in producing alimentary hyperglycemia in rabbits. They believed the ability of the various carbohydrates to cause hyperglycaemia varied directly with their rates of absorption and inversely with their ability to form glycogen and the ease with which they were oxidized. The tolerance for the different carbohydrates, starch excepted, was reduced by fasting. Factors stimulating the glucose-removing mechanism of the blood were discussed.

It is interesting to note that quite different results have been obtained when smaller doses of sugar were fed to fasting subjects. For example, it has been reported that after feeding 20 gm. of glucose, fructose, sucrose, lactose and galactose, the hyperglycemia was most marked with glucose and least with galactose, although a large amount of the latter was excreted in the urine. Starch (20 gm.) had no glycemic effect. Inasmuch as only single observations were made with some of the sugars, too much reliance should not be placed on the results.

Rowe ('24) selected galactose as the most satisfactory of the sugars to use for tolerance tests because small quantities were required and no digestive disturbances followed. The tolerance dose for men was found to be 30 gm. and for women 40 gm. This was explained on the basis of a difference between the galactose metabolism of man and woman.

Having noted that the native races of Natal regularly ingested huge quantities of sweets, May ('28) determined tolerance curves after giving 50 gm. glucose and found them normal. He then gave nine men 1000 gm. of pure glucose. All showed glycosuria which persisted for as long as 18 hours after the dose of sugar. The blood sugars with 1000 gm. glucose were higher than with 50 gm. in only three cases and in all

but three, blood sugar was approaching the normal level after 2 hours. Four of the nine showed only a slight rise in blood sugar after ingestion of the huge quantity, yet exhibited glycosuria.

Koehler, Rapp and Hill ('35) recently compared the glycemic responses of normal subjects after the ingestion of 80 to 120 gm. of lactose, starch and glucose. Lactose had no hyperglycemic effect, this having been noted earlier by Field ('19), Folin and Berglund ('22) and others. Lactosuria was not uncommon. The ingestion of starch and glucose resulted in rises in blood sugar of about the same magnitude, a peak being reached after 1 hour. The glucose curve fell more rapidly than the starch, also with the former, the level fell below the original shortly after the second hour, but the total hyperglycemia after starch was greater than for glucose.

A most interesting series of experiments with sixty infants 2 to 10 days of age, was reported by Greenwald and Pennell ('30). Distinct differences in curves for blood sugar levels of infants and adults were revealed following ingestion of sugars. After glucose intake, curves of adults and infants were practically parallel, except that those for the latter were considerably lower. The difference in response to various sugars was most striking, for blood sugar rose to practically the same level with infants following glucose, sucrose, lactose, or dextrimaltose, a response totally different from that of adults.

Using intravenous injection in contrast to ingestion, Wierzechowski, et al. ('31) found when glucose, fructose and galactose were given at a rate of 2 gm. per kilo per hour to well-nourished fasting dogs that blood sugar rose tremendously with galactose 800 mg. per cent, with glucose to 300 mg. per cent and fructose 260 mg. per cent. About 10 per cent of the total amount of glucose and fructose injected was lost in the urine, while some 70 per cent of the galactose was so lost. Injections of insulin simultaneously with the three sugars mentioned, reduced the glycemic action of glucose and galactose, but had no effect on fructosemia.

It is evident from the preceding discussion that several factors are responsible for the variable effects of different

carbohydrates on blood sugar levels. The rate of digestion and absorption, as well as the rate of passage through the alimentary tract, are of importance.

#### UTILIZATION OF CARBOHYDRATES

A preferential utilization of various carbohydrates is shown from the embryonic to the mature state of the organism. The presence of lactose in milk, and the larger quantity in human than in cow's milk, affords an interesting point of discussion. Many papers on this subject are theoretical, yet others present evidence as to the real importance of lactose for the nourishment of the young.

It is thought by some investigators that the high lactose content of human milk may be correlated with the very much greater brain development of young human beings. There is a very rapid myelinization of the fibers of the brain occurring shortly after birth during the first 6 weeks of life, this requiring extra galactose for cerebroside (galactoside) formation. The special need of this sugar in the diet of infants, has also been ascribed by some to its power of maintaining a normal intestinal flora, non-putrefactive in type. Bergeim ('26) (see also Kline, et al., '32) found that by feeding lactose to young animals, calcium and phosphorus absorption from the intestine was increased. The favorable effect noted was thought to be due to increased lactic acid formation which resulted in a more marked acidity of the feces.

As a result of observations with some 1000 children, Jarvis ('30) recommended that extra lactose should be given all infants receiving cow's milk. Extra quantities of 10 gm. at 3-day intervals were well tolerated. Babies receiving extra lactose were found to have better resistance to infection, firmer tissues, also more freedom from constipation and other nutritional and nervous upsets than a group not receiving additional lactose.

The relationship between growth and the carbohydrate ingested has been investigated by several workers. Mitchell ('27) fed rats entirely adequate diets containing 60 per cent of one of the following carbohydrates; starch, dextrin, lactose,

sucrose or maltose. The growth of the rats was normal or above for all except lactose, while starch and dextrin produced the most rapid weight increases. The diarrhea accompanying lactose ingestion prevented its utilization. If, however, the diets contained 30 per cent or less of lactose, the balance of the carbohydrate being made up by corn starch, growth was normal. From studies of growth rates of young rats, Jarvis ('30) stated that an associate in unpublished work found that those receiving lactose as the exogenous source of carbohydrate gained at a slower rate than sucrose fed animals (16 per cent less). There was significantly less water and fat in the tissues of the lactose than in the sucrose fed animals.

Ariyama and Takahasi ('29) fed young rats a carbohydrate-free basal diet, and supplemented it by adding equivalent weights of each of several carbohydrates so that the diet contained 30 per cent. The growth of the rats was followed for periods of 40 to 70 days, after which the weight gain in grams per day due to each carbohydrate was calculated. The gains in grams per day were as follows: maltose 1.63, fructose 1.38, starch 1.34, glucose 1.31, dextrin 1.28, lactose 1.27, sucrose 1.16, inulin 1.14, galactose 0.86 and carbohydrate-free 0.89. No explanations of the findings were offered. Koehler and Allen ('34) determined the relative nutritive values of glucose, sucrose and lactose by noting the gain in weight of rats after the sugars were added in equivalent amounts at 35 per cent levels to sub-maintenance diets. After 5 days, the continued weight gain was materially higher with glucose and sucrose than with lactose, due to the poor utilization of the last.

In the studies dealing with the relationship between growth and the carbohydrate ingested, ad libitum feeding experiments were used and conclusions drawn from them must not be interpreted as strictly correct.

Marked differences in the utilization of several simple sugars by various animal organs and tissues, including nerves, have been observed. Cori ('31) has reviewed factors influencing carbohydrate utilization by man and animal and a discussion of these will be omitted.

## COMBUSTION

Many workers have measured sugar utilization by noting changes in the R.Q. following ingestion of various sugars. Higgins ('16) found the respiratory quotient rose rapidly following ingestion of levulose, sucrose, or lactose, although with lactose the rise was not so marked. With glucose and maltose, a definite rise did not occur for 20 to 30 minutes. These results have been confirmed by the data of other workers published more recently. Bornstein and Holm ('22) and Holm ('23) published the results of a number of observations obtained following the ingestion of glucose, fructose and lactose by man (dosage 100 gm.) and animal (dosage 35 gm.). The blood sugar following glucose rose immediately, although burning did not begin for some 30 to 90 minutes. The blood sugar rise following fructose or lactose was negligible, but fructose began to burn within 5 to 8 minutes and lactose some 60 minutes after ingestion. The R.Q. with fructose rose higher (frequently 1.0 or over) than with either glucose or lactose. From these data, one can see that there would be little parallelism between blood sugar levels and the height of the R.Q. The authors believed that glucose was changed to a fructose-like substance prior to its combustion in the body.

Very complete studies of the effects of sugar ingestion have been made by Carpenter and his associates. Carpenter and Fox ('30 a) proved the necessity of using 200 cc. or less fluid for the ingestion of dissolved carbohydrates as larger quantities of water increased carbohydrate metabolism as much as 42 per cent and heat production as much as 7 per cent. Continuing their work ('30 b, '30 c) the respiratory exchange of a human subject in the post absorptive state was determined for 3 to 4.5 hours following the ingestion of 5 to 104 gm. of either glucose or fructose. With glucose the R.Q. increased 4 to 6 points reaching a maximum usually the third half hour following ingestion. With fructose, especially the larger quantities, the R.Q. rose more rapidly and to higher levels than noted with glucose. With glucose the heat production averaged 1 calorie for each additional 5 gm. from 5 to 25 gm.,



while in larger amounts it rose more rapidly and in proportion to the additional quantity given. Experiments with levulose revealed increases in heat production in all groups, these varying from 0.5 calorie in 1 hour after 5 gm. levulose to 46 calories in 4 hours after 10 gm.

The effects of galactose ingestion were reported by Carpenter and Lee ('32 a, '32 b). On account of the low threshold, amounts of galactose ranging from 5 to 40 gm. were fed and even with the smaller doses, there was a galactosuria. All amounts caused the R.Q. to rise, the maximum rise in a period varying from 0.04 with 5 gm. to 0.17 with 40 gm. Galactose resembled fructose in its effects on the respiratory quotient and glucose in its effects on heat production. Feeding 20- and 30-gm. doses of galactose to men and women revealed that females had higher maximum rises in respiratory quotient, greater increases in heat production at first after 20 gm., and wider individual deviations in all the features observed. On the other hand, males showed a greater increase in carbohydrate combustion and heat production after 30 gm. or more of the carbohydrate. (See also Deuel, et al., '34.)

Roe, Gilman and Cowgill ('35) pointed out that the species difference in galactose metabolism was due in all probability to the difference in response of the liver and kidneys of the species concerned. Dogs excreted much higher percentages of galactose in the urine than those reported in the literature for humans. The respiratory quotients of normal fasting dogs were not elevated significantly by feeding 2 gm. galactose per kilo. However, if 5 gm. per kilo were fed, there was a delayed elevation of R.Q. characteristic of carbohydrate oxidation. The authors suggest that galactose as such is not oxidized by dogs, rather that galactose is converted into glucose and the latter oxidized, thus causing a significant rise of the quotient. This idea has been supported by data submitted later.

A further study of the effects of sugars fed singly and in various combinations upon respiratory exchange and heat production was made by Carpenter and Lee ('32 c). When glu-

cose and fructose, galactose and fructose, or galactose and glucose were fed in the combinations mentioned, rises in respiratory quotients were noted which agreed with the summation of the rises obtained when the sugars were given separately. With similar combinations of galactose and glucose or fructose, however, there was a greater increase in heat than would be expected from summation effects. It was suggested that the cause might be: 1) the presence of more unabsorbed galactose in the alimentary tract when other sugars were ingested, 2) the greater formation of lactic acid in the intermediary metabolism of galactose, or 3) the smaller formation of glycogen when other glycogen formers were available. Further studies of the effects of glucose and fructose on respiratory metabolism were discussed in another paper (Carpenter and Lee, '33).

It should be noted that Campbell and Maltby ('28) pointed out the danger of using  $\text{CO}_2/\text{O}_2$  ratios as a means of measuring food transformation, combustion or storage of all carbohydrates. They found that fructose and cane sugar caused a lowering of the  $\text{CO}_2$  combining power of the blood and a rise in lactic acid, facts which would account for a stimulation of respiration and an increased elimination of  $\text{CO}_2$  and so, a high respiratory quotient. This was not true for glucose, maltose, lactose or galactose. Carpenter and Lee ('33) overruled this objection for fructose, at least, showing that the net rise in R.Q. as a result of its ingestion was due directly to the metabolism of this sugar, for the quantity of organic acids formed could not affect the carbon dioxide tension of alveolar air.

#### SPECIFIC DYNAMIC ACTION OF CARBOHYDRATES

The specific dynamic action of carbohydrates has been the subject of many investigations. The general conclusion of Deuel and Sandiford ('25) was that all sugars had approximately the same dynamic action. Lusk ('28, '31) presented a comprehensive review of this subject which has been augmented and brought up to date by Wilhelmj ('35), Carpenter

and Fox ('30 b, '30 c) and Carpenter and Lee ('32 a). For detailed information, the reader is referred to these papers.

#### GLYCOGEN FORMATION BY CARBOHYDRATES

Glycogen formation and storage by the liver have been found to be closely related to the carbohydrate fed, and a review of the older work has been presented in the paper by Feyder and Pierce ('35). A larger amount of liver glycogen was formed from fructose than from glucose according to results obtained by Lusk (1892), who injected the sugars subcutaneously into rabbits. Little glycogen was produced after similar administration of either sucrose or lactose. This raises the question as to whether sucrose must be hydrolyzed prior to building into glycogen. It is likely that hydrolysis of sucrose so given would be slow and as a result not rapidly available for glycogenesis. Rosenfeld ('00) allowed for urinary loss of ingested sugar and concluded that glycogen formation from the sugar retained was greatest for galactose, followed by mannose and glucose.

In early studies no attempt was made to correlate glycogen synthesis with the rate of removal of sugar from the alimentary tract. Sato ('23) injected 2.5 gm. of glucose per kilo intravenously into dogs. The glycogen content of the liver rose slowly reaching a maximum of 2 per cent in 3 hours. Repeated injections of the sugar caused a slower rise in glycogen but a larger total amount. More free sugar was found in the liver than in muscle and no increase in muscle glycogen was noted after the infusion.

Cori ('26) became interested in the relationship between the rate of sugar absorption and glycogenesis in rats. While the absorption of glucose from the intestine was twice as fast for fructose, approximately the same amount of glycogen per 100 gm. of liver was formed from each. Galactose, as a glycogen former, was relatively unimportant. Glycogenesis started slowly after giving the sugars, increased and during the fourth hour started to decrease. The variations in the rate of deposition were not believed by Cori ('26) to be due to an

unequal rate of absorption from hour to hour as he ('25) had shown a constancy of absorption coefficients over a period of hours. The maximum retention of the two sugars 4 hours after feeding, amounted to 17 per cent for glucose and 39 per cent for fructose. Four hours after giving rats either glucose or fructose, Cori and Cori ('28) determined the amount of each oxidized or laid down in the form of liver and body glycogen. Forty-four per cent of the absorbed glucose was oxidized, 18 per cent converted into liver glycogen and 25 per cent into body glycogen. With fructose 36 per cent was oxidized, 38 per cent deposited as liver and 12 per cent as body glycogen. These results showed clearly the necessity of determining glycogen not only in the liver but also in other tissues.

Feeding cornstarch or sucrose at 60 per cent levels in an otherwise balanced diet, Greisheimer and Johnson ('29) found more liver glycogen formed from sucrose than from starch. Further, replacement of part of the sucrose by casein or lard resulted in lessened glycogen deposition. Using diets containing 87.5 per cent of lactose, glucose or sucrose, they ('30) noted the percentage of liver glycogen formed from glucose and sucrose was practically the same, 4.89 per cent, and from lactose but 3.15 per cent. They concluded that if lactose were as readily digested and absorbed as the other two sugars, glycogenesis from all three would be equal. The marked ability of sucrose, as compared with other types of foods, to form glycogen was shown by the results of Greisheimer ('31).

Comparing the rates of liver glycogen formation by glucose, sucrose and corn syrup, and using the Cori ('25) technic, Feyder and Pierce ('35) noted the formation from glucose and sucrose was practically the same during the first and second hours after administration of the sugars, but in the third hour, sucrose formed approximately 44 per cent more glycogen than dextrose. This is in marked contrast with the results of Lusk (1892). Glycogenesis from corn syrup was lower than for the other two sugars, but it proceeded at a fairly uniform rate.

The superiority of sucrose as a glycogen former was assigned to the fructose portion of the molecule. The rate of deposition was independent of the rate of absorption of the sugars.

Although MacKay and Bergman ('33 a) noted no effect of previous diet on glycogen formation in rats fasted 24 or 48 hours before giving glucose, the results of Abelin ('35) showed clearly that state of nutrition was closely related to the amount of glycogen formed from sucrose or starch. There was always a more marked glycogenesis from carbohydrate when the previous diet had been rich in fat.

Recently May ('34) reported that snails, mussels, fish, lower mammals and man contained two animal starches, glycogen and galactogen, the latter being a polymer of galactose. The polysaccharide was not found to any extent in the liver, brain, ovaries or mammary glands, yet was found in large amounts in other tissues of the body. While galactogen was thought to be the mother substance of lactose and brain galactose, the author was not certain as to the sugar giving rise to galactogen. Work of other authors was cited showing that galactogen, or something similar to it, had been found in animal tissues. The significance of the polysaccharide was discussed by May, and his observations open an interesting field for investigation.

#### FAT FORMATION FROM CARBOHYDRATES

The ability of the body to store glycogen is limited and when carbohydrates are ingested in abundant quantities over a period of time another disposition is made of them, namely, fat formation. The extensive deposits of fat in the adipose tissues of meat animals and the large amounts of milk fat produced by cows, cannot be accounted for as arising solely from the food fat and as far back as the time of Liebig, carbohydrates were considered to be the most suitable raw material for fat synthesis in the animal body.

Many theories were formulated to explain the mechanism of fat formation, some claiming a direct conversion of sugar into fat, others a conversion of sugar into glycogen and a subse-

quent transformation of this storage form into fat. As pointed out by Feyder ('35), several workers have presented data which lead to the inference that glycogen is a mother substance of fat. If this is true, the question arises as to whether the glycogen-forming power of the carbohydrate is in any way related to its fat-forming power. Early work by Rubner (1885) with fasting dogs, showed that to replace 100 gm. of fat, 232 gm. of starch, 234 gm. of sucrose, or 256 gm. of glucose would be required. According to data published by Kellner ('09) starch was a more efficient source of fat than sucrose, 1000 gm. of the former giving rise to 248 gm. whereas an equal amount of the latter produced only 188 gm. The natural conclusion from the above data was that starch and sucrose were equal in fuel value but quite different in their capacity to form fat.

Discussing the ability of sugars to form fat, von Wendt ('23) believed a ketose should not be so effective as an aldose in forming fat because only 3 C atoms would be available. On the other hand, if the modified Magnus-Levy equation (Lusk, '15, '28, p. 350) is used, 4 or 6 carbon atoms would be available.

From the results obtained with an infant, Steuber and Seifert ('28) found that no fat was formed from lactose on a fat-free diet and decided that lactose was not a suitable biological substitute for fat. Later, Jarvis ('30), using rats, published results showing that sucrose formed considerably more fat than lactose and that the latter gave rise to more living tissue. Essentially the same conclusions were drawn by Whittier, Cary and Ellis ('35) from work on rats and pigs.

On comparing the increase in weight of rats in paired feeding experiments giving sucrose or glucose, Feyder ('35) noted more rapid gains after sucrose than after glucose. Further, sucrose fed animals on analysis, contained considerably more fat, but on the average slightly less glycogen than those receiving glucose. As pointed out, it was not probable that the greater fattening effect was due to a lower specific dynamic action, since fructose was known to have a greater

effect on heat production than glucose. Furthermore, Carpenter and Fox ('30) found a higher R.Q. following fructose than glucose ingestion, and believed this was due to the more rapid transformation of fructose into an oxygen poor substance, such as fat. Whether sucrose disposes the animal to reduced activity or glucose stimulates to greater activity, remains to be seen.

### SUMMARY

The absorption of carbohydrates from the stomach and intestine of man and animal has been considered. Factors influencing their removal have been presented. The literature reveals that the several sugars have quite different assimilation limits and effects upon blood sugar levels. Likewise, respiration studies demonstrate marked variations in the ease with which the body burns various carbohydrates. The formation of glycogen and fat after the administration of carbohydrates to man and animal has been reviewed.

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